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(71) Applicants:
- **SMITHKLINE BEECHAM BIOLOGICALS S.A.**
  1330 Rixensart (BE)
- **INSTITUT PASTEUR**
  75724 Paris Cédex 15 (FR)

(72) Inventors:
- **Cohen, Joe,**
  Smithkline Beecham Biologicals s.a.
  1330 Rixensart (BE)
- **Druilhe, Pierre**
  75724 - Paris Cedex 15 (FR)

(74) Representative:
Tyrrell, Arthur William Russell, Dr. et al
GlaxoSmithKline
Corporate Intellectual Property (CN9.25.1)
980 Great West Road
Brentford, Middlesex TW8 9GS (GB)

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(54) Immunogenic compositions comprising liver stage malarial antigens

(57) A vaccine composition comprising a Th1-inducing adjuvant in combination with a protecting Liver Stage Antigen or immunological fragment thereof of a human malaria parasite, especially Plasmodium falciparum, with the proviso that when the immunological fragment is an immunological fragment of LSA-3 the Th1-inducing adjuvant is not Montanide. In a preferred aspect the Th1-inducing adjuvant comprises QS21, De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition: a metabolisible oil, such a squalene, alpha tocopherol and tween 80. In a further preferred aspect the protecting Liver Stage Antigen is Liver Stage Antigen 3 (LSA-3) or an immunological fragment thereof. A multivalent vaccine composition is also provided comprising the vaccine composition of the invention and in addition at least one other protecting antigen or an immunological fragment thereof, of a malaria parasite.

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Description

[0001] The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention relates to a malaria antigen known as Liver Stage Antigen 3 in association with an oil in water emulsion. Such emulsions comprise tocopherol, squalene, Tween 80, Span 85 and Lecithin and have useful adjuvant properties. Vaccines containing QS21, an Hplc purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina, and/or 3 De-O-acylated monophosphoryl lipid A (3 D-MPL), together with such oil in water emulsions also form part of the invention. Other aspects of the invention are described hereinbelow.

[0002] It has long been known that enterobacterial lipopolysaccharide (LPS) is a potent stimulator of the immune system, although its use in adjuvants has been curtailed by its toxic effects. A non-toxic derivative of LPS, monophosphoryl lipid A (MPL), produced by removal of the core carbohydrate group and the phosphate from the reducing-end glucosamine, has been described by Ribi et al (1986, Immunology and Immunopharmacology of bacterial endotoxins, Plenum Publ. Corp., NY, p407-419).

[0003] A further detoxified version of MPL results from the removal of the acyl chain from the 3-position of the disaccharide backbone, and is called 3-O-Deacylated monophosphoryl lipid A (3D-MPL). 3 De-O-acylated monophosphoryl lipid A is known from GB2 220 211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana. GB 2122204B also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4): 392-6; Hilgers *et al.*, 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1).

[0004] A preferred form of 3 De-O-acylated monophosphoryl lipid A (3D-MPL) is in the form of an emulsion having a small particle size less than 0.2μm in diameter, disclosed in International Patent Application No. WO 92/116556 (SmithKline Beecham Biologicals s.a.). See also WO 94/21292.

[0005] Aqueous formulations comprising monophosphoryl lipid A and a surfactant have been described in WO98/43670A2.

[0006] Saponins are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). Saponins are steroid or triterpene glycosides widely distributed in the plant and marine animal kingdoms. Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating cholesterol. When saponins are near cell membranes they create pore-like structures in the membrane which cause the membrane to burst. Haemolysis of erythrocytes is an example of this phenomenon, which is a property of certain, but not all, saponins.

[0007] Saponins are known as adjuvants in vaccines for systemic administration. The adjuvant and haemolytic activity of individual saponins has been extensively studied in the art (Lacaille-Dubois and Wagner, *supra*). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst,* 1996, 12 (1-2): 1-55; and EP 0 362 279 B1. Particulate structures, termed Immune Stimulating Complexes (ISCOMS), comprising fractions of Quil A are haemolytic and have been used in the manufacture of vaccines (Morein, B., EP 0 109 942 B1; WO 96/11711; WO 96/33739). The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No.5,057,540 and EP 0 362 279 B1. Other saponins which have been used in systemic vaccination studies include those derived from other plant species such as Gypsophila and Saponaria (Bomford *et al.*, Vaccine, 10(9):572-577, 1992).

[0008] QS21 is a Hplc purified non toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.

[0009] Oil emulsion adjuvants have been known for many years, including work on Freund's complete and incomplete mineral oil emulsion adjuvants. Since that time much work has been performed to design stable and well tolerated alternatives to these potent, but reactogenic, adjuvant formulations.

[0010] Many single or multiphase emulsion systems have been described. Oil in water emulsion adjuvants *per se* have been suggested to be useful as adjuvant compositions (EP O 399 843B), also combinations of oil in water emulsions and other active agents have been described as adjuvants for vaccines (WO 95/17210). Other oil emulsion adjuvants have been described, such as water in oil emulsions (US 5,422,109; EP 0 480 982 B2) and water in oil in water emulsions (US 5,424,067; EP 0 480 981 B).

[0011] In order for any oil in water composition to be suitable for human administration, the oil phase of the emulsion system preferably comprises a metabolisable oil. The meaning of the term metabolisable oil is well known in the art. Metabolisable can be defined as "being capable of being transformed by metabolism" (Dorland's Illustrated Medical Dictionary, W.B. Sanders Company, 25th edition (1974)). The oil may be any vegetable oil, fish oil, animal oil or synthetic oil, which is not toxic to the recipient and is capable of being transformed by metabolism. Nuts (such as peanut oil), seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this invention and can include commercially available oils such as NEOBEE® and others. Squalene (2,6,10,15,19,23-Hexamethyl-

2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil which is found in large quantities in shark-liver oil, and in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil virtue of the fact that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no.8619).

[0012] The oil in water emulsions which form part of the present invention when formulated with 3 D-MPL and QS21 are preferential stimulators of IgG2a production and TH1 cell response. This is advantageous, because of the known implication of TH_1 response in cell mediated response. Indeed in mice induction of IgG2a is correlated with such an immune response.

[0013] The observation that it is possible to induce strong cytolytic T lymphocyte responses is significant as these responses, in certain animal models have been shown to induce protection against disease.

[0014] The present inventors have shown that the combination of the adjuvants QS21 and 3D-MPL together with an oil in water emulsion with an antigen results in a powerful induction of CS protein specific CTL in the spleen. QS21 also enhances induction of CTL on its own, while 3D-MPL does not.

[0015] Induction of CTL is easily seen when the target antigen is synthesised intracellularly (e.g. in infections by viruses, intracellular bacteria, or in tumours), because peptides generated by proteolytic breakdown of the antigen can enter the appropriate processing pathway, leading to presentation in association with class I molecules on the cell membrane. However, in general, pre-formed soluble antigen does not reach this processing and presentation pathway, and does not elicit class I restricted CTL. Therefore conventional non-living vaccines, while eliciting antibody and T helper responses, do not generally induce CTL mediated Immunity. The combination of the two adjuvants QS21 and 3D-MPL together with an oil in water emulsion can overcome this serious limitation of vaccines based or recombinant proteins, and induce a wider spectrum of immune responses.

[0016] CTL specific for CS protein have been shown to protect from malaria in mouse model systems (Romero et al. Nature 341:323 (1989)). In human trials where volunteers were immunised using irradiated sporozoites of P. falciparum, and shown to be protected against subsequent malaria challenge, induction of CTL specific for CS epitopes was demonstrated (Malik et al. Proc. Natl. Acad. Sci. USA 88:3300 (1991)).

[0017] The ability to induce CTL specific for an antigen administered as a recombinant molecules is relevant to malaria vaccine development, since the use of irradiated sporozoites would be impractical, on the grounds of production and the nature of the immune response.

[0018] In certain systems, the combination of 3D-MPL and QS21 together with an oil in water emulsion have been able to synergistically enhance interferon γ production.

[0019] IFN-γ secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of macrophages by IFN-γ enhances intracellular killing of microbes and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another product of TH1 cells). IFN-γ is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN-γ or other mechanisms, provide preferential help for IgG2a immunoglobulin isotypes.

[0020] RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of P.falciparum linked via four amino acids of the $preS_2$ portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus (HBV). The structure of RTS and the molecules from which it is derived is disclosed in International Patent Application Publication Number WO 93/10152. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S.

[0021] Liver Stage Antigens are described in Malaria, Parasite Biology, Pathogenesis and Protection (1998 ASM Press, Washington D.C., edited by Irwin W. Sherman), especially Chapter 34 (P. Druilhe et al.).

[0022] A 26-amino acid synthetic peptide based on Plasmodium falciparum liver stage antigen 3 (LSA-3) is described in Eur J. Immunol., 1997, 27, 1242-1253 (L. BenMohamed et al).

[0023] The immunogenicity of 12 synthetic peptides derived from four new Plasmodium falciparum molecules expressed at pre-erythrocytic stages of the human malaria parasite was reported in Vaccine 18 (2000), pages 2843-2855 (L BenMohamed et al). In these studies the adjuvant Montanide ISA-51 (SEPPIC, Quai D'Orsay, France) was used. There is no report, however, of such peptides being combined with other adjuvants. The present invention is based on the surprising discovery that a Th-1 inducing adjuvant especially an oil in water emulsion which preferably comprises tocopherol, as such or in combination with QS21 and/or 3 D-MPL (or related molecules), enhances immune responses to a defined malaria antigen. Such enhancement available affords better immunological responses than hitherto before.

[0024] According to the present invention there is provided a vaccine composition comprising a Th1-inducing adjuvant in combination with a protecting Liver Stage Antigen or immunological fragment thereof of a human malaria parasite with the proviso that when the immunological fragment is an immunological fragment of LSA-3, the Th1-inducing adjuvant is not Montanide.

[0025] In a preferred aspect of the invention the Th1-inducing adjuvant comprises QS21, De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition:

a metabolisible oil, such a squalene, alpha tocopherol and tween 80.

[0026] It will be appreciated that variants or derivatives of QS21 and 3-DMPL as described above may also be used without departing from the spirit of the invention.

[0027] The bacterial lipopolysaccharide derived adjuvants to be formulated in the adjuvant combinations of the present invention may be purified and processed from bacterial sources, or alternatively they may be synthetic. Accordingly, the LPS derivatives that may be used in the present invention are those immunostimulants that are similar in structure to that of LPS or MPL or 3D-MPL. In another aspect of the present invention the LPS derivatives may be an acylated monosaccharide, which is a sub-portion of MPL. In a preferred aspect the 3-DMPL is small particle 3-DMPL as described in WO 92/116556.

[0028] The oil emulsion adjuvants for use in the present invention may be natural or synthetic, and may be mineral or organic. Examples of mineral and organic oils will be readily apparent to the man skilled in the art based on the description hereinabove.

[0029] Particularly preferred oil emulsions are oil in water emulsions, and in particular squalene in water emulsions.

[0030] In addition, the most preferred oil emulsion adjuvants of the present invention comprise an antioxidant, which is preferably the oil α-tocopherol (vitamin E, EP 0 382 271 B1).

[0031] WO 95/17210 discloses emulsion adjuvants based on squalene, α-tocopherol, and TWEEN 80, optionally formulated with the immunostimulants QS21 and/or 3D-MPL.

[0032] The size of the oil droplets found within the stable oil in water emulsion are preferably less than 1 micron, may be in the range of substantially 30-600nm, preferably substantially around 30-500nm in diameter, and most preferably substantially 150-500nm in diameter, and in particular about 150 nm in diameter as measured by photon correlation spectroscopy. In this regard, 80% of the oil droplets by number should be within the preferred ranges, more preferably more than 90% and most preferably more than 95% of the oil droplets by number are within the defined size ranges. The amounts of the components present in the oil emulsions of the present invention are conventionally in the range of from 2 to 10% oil, such as squalene; and when present, from 2 to 10% alpha tocopherol; and from 0.3 to 3% surfactant, such as polyoxyethylene sorbitan monooleate. Preferably the ratio of oil: alpha tocopherol is equal or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of about 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser. Preferably the oil emulsion contains a surfactant such as polyoxyethylene sorbitan monooleate (TWEEN80™), but it will be clear to the man skilled in the art that other surfactants may be used, preferred examples of which are the SPAN series (especially SPAN85) and or lecithin.

[0033] The method of producing oil in water emulsions is well known to the man skilled in the art. Commonly, the method comprises the mixing the oil phase with a surfactant such as a PBS/TWEEN80™ solution, followed by homogenisation using a homogenizer, it would be clear to a man skilled in the art that a method comprising passing the mixture twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in microfluidiser (M110S microfluidics machine, maximum of 50 passes, for a period of 2 minutes at maximum pressure imput of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion. This adaptation could be achieved by routine experimentation comprising the measurement of the resultant emulsion until a preparation was achieved with oil droplets of the required diameter.

[0034] In a preferred aspect of the invention the human malaria parasite is Plasmodium falciparum.

[0035] In a particular aspect of the invention the said protecting Liver Stage Antigen is the Liver Stage Antigen 3 (LSA-3) or immunological fragment thereof.

[0036] However other Liver Stage Antigens may also be used, for example LSA-1 and LSA-2 as described in Malaria, Parasite Biology, Pathogenesis and Protection (1998 ASM Press, Washington D.C., edited by Irwin W. Sherman), especially Chapter 34 (P. Druilhe et al.).

[0037] By immunological fragment is meant herein a molecule which has a related or similar sequence to the reference antigen in terms of % homology and which can induce a similar immune response, cellular or humoral, in vivo.

[0038] The LSA-3 antigen and polypeptide molecules containing at least 10 consecutive amino acids of the amino acid sequence representing LSA-3 are described in WO 96/41877. LSA-3 for use in the present invention may suitably be prepared as described in the examples section of the present specification. Reference may also be made to C Marchand and P Druilhe, Bulletin of the World Health Organisation, Volume 68 (Suppl.) 158-164 (1990) and US Patent Number 6,100,067.

[0039] In a further aspect there is provided a vaccine composition according to the invention comprising in addition at least one other protecting antigen or an immunological fragment thereof, of a malaria parasite, in particular LSA-3.

[0040] In particular, the other malaria antigen may be selected from the following group:

a) a hybrid protein comprising substantially all the C-terminal portion of the CS protein, four or more tandem repeats of the immunodominant region, and the surface antigen from hepatitis B virus (HBsAg), in particular RTS,S, or an immunogenic derivative including fragments thereof;

b) the TRAP protein of the T9/96 isolate of Plasmodium falciparum and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof (see European Patent Application No 91903249.0);

c) the MSP-1 of Plasmodium falciparum or Plasmodium vivax and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof; and

d) the MSP-3 of Plasmodium falciparum or Plasmodium vivax and proteins having at least 70% homology with the C-terminal region thereof, and immunogenic derivatives including fragments thereof.

[0041] MSP-1 of P.falciparum or P.vivax is described in US Patent No. 4,837,016. Immunogenic derivatives include fragments thereof such as the C-terminal 42 KDa antigen (p42).

[0042] The MSP-3 antigen is described in US Patent Number 6,017,538.

[0043] Homology in sequence analysis may be established by the use of Blast 2.0 and Fasta default settings of the algorithms used by these programs. The comparison of LSA-3 sequences in various isolates or stocks can be done using a calculation manual.

[0044] By C-terminal region of MSP-3 is meant a 185 amino acid region from positions 193 to 381. It contains a leucine zipper on its extremity (C-terminus part) and is rich in acidic amino acids. The three-dimensional structure is coil-coiled. The clone DG 210 (amino acids 193-257) corresponds to a globular region of high complexity and is followed by the coil-coiled region.

[0045] Normally the vaccine composition according to any aspect of the invention invokes a T cell response in a mammal to the antigen or antigenic composition and is preferably capable of stimulating interferon γ production.The oil in water emulsion used in the present invention may be utilised on its own or with other adjuvants or immunostimulants and therefore an important embodiment of the invention is an oil in water formulation comprising squalene or another metabolisable oil, alpha tocopherol, and tween 80. The oil in water emulsion may also contain span 85 and/or Lecithin.

[0046] The combination of the two adjuvants QS21 and 3D-MPL together with an oil in water emulsion is particularly preferred. This is known and referred to herein as SBAS2.

[0047] The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D MPL: QS21. Typically for human administration QS21 and 3D MPL will be present in a vaccine in the range 1 μg - 100 μg, preferably 10 μg - 50 μg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. Preferably the ratio of squalene: alpha tocopherol is equal or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

[0048] In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

[0049] In yet a further aspect the invention provides a process for making a vaccine composition according to any aspect of the present invention by mixing the required components using standard techniques. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

[0050] Preferably the process comprises admixing QS21, 3D-MPL and the oil in water emulsion with a protecting Liver Stage Antigen of a human malaria parasite as hereinabove defined, optionally with an additional malaria antigen.

[0051] The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000ug of protein, preferably 2-100 ug, most preferably 4-40 ug. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

[0052] The formulations of the present invention maybe used for both prophylactic and therapeutic purposes.

[0053] Accordingly in one aspect, the invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient.

[0054] The following examples illustrate the invention.

Examples

Example 1

[0055] Two adjuvant formulations were made each comprising the following oil in water emulsion component.

[0056] SB26: 5% squalene 5% tocopherol 0.4% tween 80; the particle size was 500 nm size SB62: 5% Squalene 5% tocopherol 2.0% tween 80; the particle size was 180 nm

1(a) Preparation of emulsion SB62 (2 fold concentrate)

[0057] Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

1(b) Preparation of emulsion SB26

[0058] This emulsion was prepared in an analogous manner utilising 0.4% tween 80.

[0059] To the emulsion of 1 a) or b) an appropriate amount of LSA-3 (for example 2μg to 100μg) may be added and mixed. This may be combined with, for example, 50μg/ml of 3D-MPL and 20μg/ml of QS21 (or related molecules) to give the final formulation.

Example 2

Protection against *Plasmodium falciparum* malaria in chimpanzees by immunisation with a conserved pre-erythrocytic antigen, LSA-3

[0060] The basis of the strong immunological protection induced in humans by vaccination with radiation-attenuated pre-erythrocytic malaria parasites is poorly understood. However it is now suspected that the transformation of the irradiated sporozoites into live but developmentally arrested intra-hepatic liver trophozoites is required to induce protection[9]. This occurs at low (15-20 krad) but not at high (23-30 krad) irradiation doses[9,10]. We reasoned that the differential response of hosts immunised with such irradiated sporozoites could provide a screen for molecules relevant to protection. We proceeded to screen 120 phage lambda clones previously identified as expressing *P. falciparum* polypeptides that are expressed during pre-erythrocytic stage parasite development[6,7] and which derive from ca. 20 distinct genes[6,7,11,12]. A clone corresponding to each of these putative genes was screened using eight sera from human volunteers (4/6 protected) and from chimpanzees (1/2 protected) immunised with sporozoites irradiated at low or high doses. A single clone (DG729) reacted only with sera from protected humans and chimpanzees. This differential reactivity was further confirmed with a peptide derived from this fragment (Table I). This led us to select this clone for further investigation.

[0061] DG729 was used to probe a *P. falciparum* (K1) genomic library. One clone was found to contain the whole gene corresponding to DG729, and which was named Liver Stage Antigen-3 (LSA-3). Full description of the sequence, expression, location and conservation of the *lsa-3* gene is provided in the Supplementary Information (S.I.) and is summarised below and in Figures 1-3. Briefly we identified a single-copy gene which comprises a mini-exon 1, a mini-intron, and a large exon 2 (Fig. 1a), a structure similar to that of other surface antigens of *P. falciparum*[13]. It was recently confirmed that *lsa-3* is located on chromosome 2[14], where the gene was annotated as « RESA-H3 » gene (Acc. Number AE001424). LSA-3, with a predicted molecular weight of 200 kDa (in K1), is made up of large non-repeated sequences flanking three glutamic acid-rich repeated regions, a feature that extends the known *P. falciparum* Glu-rich antigen network[15] to include a pre-erythrocytic component. The location of the original fragment (DG729) and of the peptides corresponding to the repeat region R2 and to the non-repetitive regions NR-A and NR-B are shown in Fig. 1b. Naturally- or artificially- induced antibodies against the non-repeated peptides and the recombinant protein GST-PC were not cross-reactive with the repeated Glu-rich regions, and were used for further studies.

[0062] Pre-erythrocytic expression of LSA-3 (see Fig. 2-3 and see S.I.) was confirmed a) by RT-PCR (primers i1 and i2) of total RNA and Western blotting of protein extracts, isolated in both cases from sporozoites, and b) by immunofluorescence antibody test (IFAT) on infected liver sections and dry or wet sporozoite preparations, using antibodies to a non-crossreactive portion of the protein. In the five and six day-old liver schizonts, LSA-3 was located in the parasitophorous vacuole and at the periphery of maturing hepatic merozoites. This location is consistent with the molecular structure of this protein, which contains two hydrophobic regions (Fig. 1a). In our hands, mRNA from *lsa-3* could not be detected in Northern blotted RNA from erythrocytic stages. Western blottings and IFAT of infected red blood cells were also consistently negative with non cross-reactive antibodies. Reactivity was however obtained when antibodies to the Glu-rich repeat region were used. This might explain in part the detection of a putatively homologous antigen (D260) previously described in intra-erythrocytic parasites, and which was identified solely using antibodies which cross-react extensively with Glu-rich epitopes[16].

[0063] Polymorphism of many malaria vaccine candidate molecules is of recognised concern, we therefore investigated naturally occurring sequence variation in LSA-3 (see S.I.). The gene was consistently detected by PCR amplification of the NR-A region (primers S1 and S2) in a total of 111 *P. falciparum* isolates, strains or clones of various

geographical origin. Using LSA-3 specific antibodies in IFAT assays, the expression of LSA-3 was also detected in liver schizonts of two distinct strains and in all the sporozoites from 30 wild isolates which developed in mosquitoes fed *in vitro* on Thai gametocytes. The repeat regions R1 and R3 are highly conserved, but variation in the number and order of the repeat units of R2 was found to occur amongst different parasite lines. This did not however affect the predicted conserved ?-helical organisation, a secondary structure considered to be important in defining major B-cell epitopes since antibodies which recognise R2 did indeed react positively by IFAT with all the parasites tested. The non-repeated portions of exon 2, where numerous Th and CTL epitopes are found[17-19], displayed a remarkable degree of amino acid (aa) sequence conservation between different parasites (>95.5% homology). The sequence of NR2 peptide was fully conserved amongst K1 and T9/96 parasites, the source of the immunising proteins, the NF54 parasites used for sporozoite challenges, and 27 *P. falciparum* samples of various geographical origin[17]. An HLA-B53 restricted epitope identified in the NR-B region of LSA-3 (present in GST-PC recombinant protein) was also found to be free of variation in clone 3D7 and in 18 Gambian isolates[19]. This conservation of immunologically important epitopes contrasts with substantial polymorphism in current pre-erythrocytic vaccine candidates.

[0064] We selected the chimpanzee to investigate the protective capacity of LSA-3 immunisation for the following reasons. The chimpanzee is the only non-human primate fully susceptible to complete intra-hepatic development of *P. falciparum*, with a comparable rate of sporozoite transformation to liver forms to that seen in humans[9]. The chimpanzee is also the most closely related animal to humans (98.4 % homology at the DNA level [8]), and one in which detailed investigations of immune responses can be performed and legitimately compared with those of humans [17,18]. The fact that parasitological and immunological events can be directly examined in the liver biopsies, a possibility excluded for infected humans, is clearly of considerable significance. A number of preliminary stringent tests were conducted in control animals in order to validate the suitability of this model for vaccine evaluation. Since cost and ethical considerations preclude the use of large number of animals, high reproducibility of the infection in this model system is critical. In a preliminary experiment (Group I, Table II), we confirmed that in the chimpanzee protection by immunisation with irradiated sporozoite is radiation dose-dependent, and we validated the detection of the infected red blood cells as an assay of protection. The results allowed us to define a number of important parameters: a) as in humans, chimpanzees develop a powerful protective response following immunisation with irradiated sporozoite, b) chimpanzees, like humans, remain broadly susceptible to at least five successive challenges, in contrast to lower primates or rodents which become refractory after the first challenge[20], and c) as a result of the high dose of inoculated sporozoites detection of erythrocytic parasites corresponded to the first invasion of red cells by merozoites released from intra-hepatocytic schizonts. Positive blood smears were reproducibly obtained in non-protected chimpanzees on days six or seven, In the chimpanzee erythrocytic infections normally remain sub-clinical and self-limiting which was in fact observed despite the high dose challenges. These results have been recently confirmed in two further chimpanzees (Langermans J. *et al*, manuscript in preparation).

[0065] Having established the suitability of the chimpanzee, we proceeded to assay the protective value of LSA-3 immunisation by challenge with viable *P. falciparum* sporozoites. In preliminary experiments, two animals were immunised with a mixture of LSA-3 and LSA-1 recombinant proteins. Full protection against three challenges over several months was only seen in the animal which responded to LSA-3 (both responded to LSA-1). In liver biopsies performed on this animal on day five, only one liver schizont of unhealthy appearance and infiltrated by leukocytes could be detected in the 300 liver sections screened (Dirk, Fig. 3). By contrast 2500 and 750 hepatic schizonts of healthy appearance were observed in the two non-protected controls.

[0066] These results led us to focus further immunisation and challenge experiments on LSA-3 alone. Two groups of chimpanzees were used to evaluate lipopeptide and recombinant protein formulations (Table II, Groups II-III). In Group II, one animal (Gerda) was initially immunised solely with the NR2 lipopeptide of LSA-3, and boosted by recombinant LSA-3 molecules in Montanide ISA 51. Gerda was fully protected when challenged with 10^7 sporozoites, whereas the control receiving Montanide ISA 51 was not (Fig. 4a).

[0067] In Gerda boosting with the recombinant LSA-3 formulation was not found to induce any detectable increase in the strong B-cell, T-helper cell and CTL responses already evoked by the initial lipopeptide/peptide injections[17,18]. We were therefore interested to see whether the simple and well-tolerated peptidic formulation alone could induce protection. Two chimpanzees, Mopia and Mgbado were immunised with LSA-3 lipopeptides/peptides alone (Table II, Group III). Protection against a first challenge with 2×10^4 sporozoites was obtained in both. The same group included an investigation of the effects of microbead presentation of recombinant proteins without adjuvant in one animal (Judy) which resulted in a one-day delay to patency (Fig. 4b). Following a subsequent high dose sporozoite challenge (5×10^6 sporozoites), both Mopia and Mgbado demonstrated a clear two-day delay to patency and a low transient parasitaemia, whilst no protection was found for Judy (Fig. 4c). The delay to patency suggests that the immune responses had caused a reduction exceeding 90% of intra-hepatocytic schizont load[21] (Fig. 4).

[0068] In chimpanzees from groups IV and V, we investigated the efficacy of a less complex lipopeptide mixture alone, or of recombinants adjuvated by SBAS2, a novel adjuvant whose efficacy has been recently established in humans[4,5]. Since immunogenicity studies[17,18] and analysis of previous chimpanzee data had indicated that peptide

CT1 was poorly immunogenic and thus might not be critical, chimpanzee Patty was immunised by a mix of three instead of four peptides. This animal showed protection upon challenge. Among four animals receiving SBAS2 adjuvated LSA-3 proteins, two showed full, sterile protection against a medium dose challenge. One showed a delay in patency which may be indicative of partial protection, whereas neither the fourth nor the control receiving SBAS2 adjuvant alone were protected. One of the two fully protected chimpanzees was further challenged with a high dose three months later and still showed full protection.

[0069] We present here the first description of protective vaccination against human malaria in the chimpanzee. This model provided us with convincing evidence that LSA-3 of *P. falciparum* is a valuable candidate for effective vaccination against pre-erythrocytic stages. A total of nine animals were immunised using lipopeptides in saline or polypeptides in either Montanide or SBAS2 adjuvants. Full sterile protection was induced in six of these nine chimpanzees on first challenge. If the significant delay as compared to controls is taken in consideration, a protective effect induced by LSA-3 was shown in eight of nine animals. Out of the 14 challenges which were performed, complete protection was obtained in seven, and partial protection in an additional four challenges. All seven control animals employed in these studies showed a consistent pattern in the appearance and the course of the blood-stage parasitaemiae following each of the 12 challenges with viable parasites. Demonstration of this reproducibility in controls, in animals immunised by over-irradiated sporozoites, and in an additional 26 challenges performed in other experiments (not shown), is an essential point in the interpretation of our data.

[0070] It is encouraging that protection was induced against a heterologous challenge (NF54) in outbred animals immunised with LSA-3 molecules whose sequences were derived from K1 and T9/96 parasites. A variety of immunisation strategies were investigated in the course of this work. The data underpin the value of the SBAS2 adjuvant. The results with Gerda, Mopia, Mgbado and Patty are also particularly encouraging since they are based on simple peptide and lipopeptide formulations which are relatively easy to produce under GMP conditions[22]. In our animals no local or general reactions was detected following lipopeptide injections, an observation consistent with previous experience with similar formulations derived from SIV in macaques[23] and HbS[24] or HIV[22] in humans. This bodes well for future clinical trials.

METHODS

[0071] **Selection of clone DG729.** Dot blot analysis of the β-galactosidase-fused recombinant proteins encoded by the pre-erythrocytic clones was performed on nitrocellulose as previously described[7], using 1/100 diluted human and chimpanzee sera. ELISA was performed in duplicate as previously described[25] on 1/100 diluted sera using coating solutions of 0.3, 3 and 10 µg/ml ofNR1, NR2 and RE peptides respectively, in PBS.

LSA-3 cloning and characterisation. Detailed description of molecular methods, gene cloning, sequence data, protein characteristics and description of the recombinant proteins and of the peptides are provided in the S.I. The primers used for PCR: S1 (nucl.161-184)/S2 (nucl.454-432) and for RT-PCR: i1 (nucl.695-722)/i2 (nucl.824-799), numbering refers to the *lsa-3* sequence of K1 (Accession Nber AJ007010). All mouse sera used for the Western blot (at dilution 1/100) presented in Fig. 2 were obtained following 3 subcutaneous injections of the immunogen (100 µg) emulsified in SBAS2 adjuvant[4]. Long synthetic peptides GP5, GP6, GP8 and GP11 were synthesised as described in ref. 26 (see Fig. 1 for position).

Immunogens injected in chimpanzees. Sequences of the various immunogens evaluated here consisted of clone DG729 and inserts NN and PC, as well as peptides (pep.) NR1, NR2, RE and CT1; their location is shown in Fig. 1 and described in more details in the S.I. Clone DG729, as well as inserts NN and PC were expressed as glutathione-S-transferase-fused recombinants and purified according to manufacturer recommendations (Invitrogen, The Netherlands). Recombinants GST-DG729, -NN and -PC were designed so as to cover 95% of the LSA-3 antigen and were used as a mixture mentioned as LSA-3 GST-rec. Peptides NR1, NR2 and CT1, were also synthesised as palmitoyl-conjugated lipopeptides (lipopep.), as described in ref. 17. Combination of synthetic compounds (mentioned as (lipo) pep.) consisted in a mixture of NR1, NR2 and CT1 lipopeptides and of RE peptide. All peptides and lipopeptides were purified to >90% purity by reversed-phase chromatography, and the impurities consisted essentially of related peptides of shorter sequences[17].

Chimpanzee immunisations and challenges. None of the chimpanzees included in this study had previously been exposed to malaria infections or malarial antigens.

Recombinant and synthetic compounds were injected subcutaneously, at a dose of 100 µg for each peptide and/or lipopeptides, and/or 50 µg for each protein. Lipopeptides were always injected in PBS and, except when mentioned, peptides and recombinants were emulsified in Montanide ISA51. Group I animals (Carl and Japie) were immunised by five intra-venous injections of 5 x 10^6 gamma-irradiated sporozoites at day 0 and weeks 8, 24, 44 and 65, and received three challenges at weeks 71, 97 and 123 (challenge doses are given in Table II). One year after the three challenges reported here, these chimpanzees were re-immunised once, and received one low and one high dose challenges, which revealed the same pattern of protection (not shown, Langermans J. *et al.*, manuscript in preparation).

In Group II, Gerda received NR2 lipopeptide at day 0 and weeks 3, 13 and 31 as described in ref. 17. She was then boosted with the mixture of LSA-3 GST-rec. at weeks 40, 45, 48 and 50. Control animal Lianne received Montanide ISA51. Challenges were performed at week 60. Group III animals were immunised at day 0 and weeks 3 and 6. Mopia and Mgbado received LSA-3 (lipo)peptides whereas Judy was injected with LSA-3 GST-rec. adsorbed to latex microbeads. Challenges LD and HD were performed at weeks 21 and 29. In Group IV, Patty received LSA-3 (lipo)peptides, but without lipopeptide CT1, whereas Wendy and Willy were injected with LSA-3 GST-rec in SBAS2 adjuvant[4,5]. Control animal Helen received SBAS2 adjuvant only. All animals were immunized at weeks 0, 4 and 8 and were challenged with 20,000 sporozoites at week 13. In Group V, Cindy and Marty were both immunised at weeks 0, 4, 8 and 26 with LSA-3 GST-rec in SBAS2 adjuvant (as in Group IV) and negative control animal Fauzi received over-irradiated sporozoites similarly to Japie (Group I) at weeks 5, 8, 11 and 26. Challenges LD and HD were performed at weeks 33 and 46 in all three animals.

NF54 sporozoites were obtained from dissected salivary glands of infected *Anopheles gambiae* as previously described[27]. Sporozoites were pooled, resuspended in PBS and injected intravenously. All animals in each group were challenged with the same pool of sporozoites. For cost reasons, extensive evaluation of the Minimal Infective Dose has not been undertaken, however challenge with 5 x 10^3 sporozoites, the lowest dose used to date, has proven infective in four other animals (Thomas, A.W., unpublished data).

Determination of the protective status. For Groups I, II, IV and V, animals blood was taken on days five to nine, and evaluated by thick and thin film Giemsa-stained preparations, and confirmed in all cases by *in vitro* culture (not shown), as described in ref. 21. For Group III chimpanzees blood taken every day from day five up to day 18, then every other day up to day 30, was used to prepare thin and thick smears which were Giemsa-stained and examined by two separate microscopists. A chimpanzee was considered a) totally protected when no parasites could be detected in the circulation blood, by direct microscopical observation and by long term culture, or b) partially protected when time to patency was delayed by one or more days as compared to that observed in control animals. In mice, these delays correspond to a protection of 80% (24h) or 96% (48h) against sporozoite challenges. In humans, a 12 hour delay was calculated to correspond to a 92% reduction of liver forms following sporozoite challenges[21]. In a limited number of animals a liver biopsy was performed under anaesthesia by a veterinary doctor on day five following a high dose challenge. Material was fixed and 4 µm sections were made and stained by Giemsa-collophonium28 before complete microscopic enumeration of the liver forms in 300 sections (average area 0.8 cm^2). All animals were curatively treated with chloroquine immediately after the period of observation, and irrespective of their protective status.

References to Example 2

[0072]

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Code or Name	Spz. irrad. dose	IFAT titers on spz.	status	NR2 peptide (aa 198-223)
V4	23.6	4,096	not protected	0.5
V5	23.6	32,000	protected	0.5
Japie	30	3,200	2 day delay not protected	0.7
V6	20.8	5,120	Protected	3.8
V7	20.8	41,960	Protected	2.6
V8	20.8	40,960	Protected	4.8
WR4	15	3,200	Protected	3.4
Carl	18	6,400	Protected	2.3

Spz.: sporozoite; irrad.: irradiation

[0073] Table I. Differential reactivity of sera from protected or non-protected humans or chimpanzees with peptide NR2. IgG-specific antibodies against peptide NR2 were measured by ELISA in sera from human volunteers (codes) and chimpanzees (names in italic) immunised with sporozoites irradiated at low or high dose (in krad). Codes, immunisation schemes, sporozoite IFAT titres and protective status determination for human volunteers V4-V8 and WR4 are detailed in ref. 1 and 2, respectively. Chimpanzees Carl and Japie were immunised and challenged as described in the text and the Methods (Group I). ELISA titres are expressed in arbitrary units representing the ratio of the mean ODs from test sera to the mean OD plus three standard deviations from 10 controls studied in parallel in the same plate. Results are taken as positive for ratios above one (in bold). Similar experiments performed with peptides NR1 and RE (see Fig. 1) yielded negative results with these sera (not shown).

ANIMAL GROUPS		Immunisation and challenge dates (weeks)	PROTECTION	
Chimp.	Immunisation protocols[a]		LD 2x10 4	HD 10^7
	Group I[b]	97 123		
Carl	18 krad-irradiated sporozoites	[0-8-24-44-65]	+	+
Japie	30 krad-irradiated sporozoites		-	-
Marcel	*unimmunised control*	60	-	-
Theo	*unimmunised control*	[0-3-15-31] [40-45-48-50]	-	-
	Group II			
Lianne	rec. in ISA51]	21 29[c] [0-3-6]	nd	-
	control ISA 51			
	Group III	13		
Mopia	[(lipo)pep.]	[0-4-8]	+	d2
Mgbado	[(lipo)pep.]	33 46	+	d2
	[GST-rec. in SBAS2]	[0-4-8-26]		
Ondele	*control GST / microbeads*	[5-8-11-26][f]	-	-
Makata	*unimmunised control*		-	-
	Group IV			
Patty	[(lipo)pep.][d]		+	nd
Wendy	[GST-rec. in SBAS2]		+	nd
Willy	[GST-rec. in SBAS2]		-	nd
Helen	*control SBAS2*		-	nd
	Group V			
Cindy	[GST-rec. in SBAS2]		+	+
Marty	[GST-rec. in SBAS2]		d1	-
Fauzi	*30 krad-irradiated sporozoites*		-	-

Chimp.: chimpanzee name; HD/LD: high/low dose sporozoite challenges; d1/d2: one/two-day delay to patency; nd: not done.

a) details and abbreviations are given in the Methods.

b) Group I chimpanzees received three additional challenges (2 LD and 1 HD) which led each time to similar results, i.e. a reproducible protection only in Carl (data not shown).

c) HD challenge was performed with 5 x 10^6 sporozoites.

d) same mixture as in Group III but without peptide CT1.

e) performed in Cindy and Marty.

f) performed in Fauzi.

[0074] Table II. Immunisation and challenge experiments in the chimpanzees. Challenges were performed with either $2x10^4$ (low dose) or 10^7 (high dose) NF54 *P. falciparum* sporozoites ("Protection" column). Immunisation schedules (in brackets under the bar) and of challenges (indicated by arrows above the bar) are expressed in weeks from first immunisation. Shading highlights protected animals. Complete protection is indicated with (+); a delay to patency (in days) as compared to controls and non-protected animals is indicated by d1 or d2 (determination of the protective status is detailed in the Methods).

LEGENDS FOR FIGURES

[0075] Figure 1: Schematic representation of the LSA-3 gene, recombinant proteins and peptides. a) 6.2 Kb *Eco* RI-insert isolated from K1 parasite genomic DNA library that hybridised with DG729. The 5.53 Kb gene comprises a 198 bp exon 1, a 168 bp intron (i) and a 5.16 Kb exon 2. Regions NR-A, -B and -C correspond to non-repeated sequences whereas R1 to R3 designate the three repeat blocks. The two hydrophobic regions potentially corresponding to the NH_2-terminal signal peptide and the anchor region are indicated by HR1 and HR2 respectively. b) Location of the sequences encoding for LSA-3 in the recombinant fusion proteins (first line) and the synthetic peptides (strokes) used in this study (see Supplementary Information for aa numbering). For the immunisations, CT1 and NR2 were also used as palmitoyl-conjugated lipopeptides[17] (indicated by *).

[0076] Figure 2: LSA-3 expression in *P. falciparum* sporozoites. Western blot analysis was performed on protein extracts from NF54 sporozoites and control uninfected mosquito salivary glands using mouse antisera directed against: C) control GST, 1) GST-PC, 2) peptides GP5, GP6, GP8 or GP11, 3) GST-729 (see Fig. 1, Methods and S.I.). LSA-3 is visualised as a 175 kDa protein (*), in agreement with the theoretical molecular weight of LSA-3 in this parasite strain.

[0077] Figure 3: Immunostaining of *P. falciparum* pre-erythrocytic stages with anti-LSA-3 antibodies. a) sporozoites stained by IFAT with human antibodies affinity purified on recombinant βga1-DG729. b) staining by IFAT of day six post-challenge liver stages[29] from a chimpanzee, using the antibodies induced by lipopeptide NR2 injection[17] in chimpanzee Gerda (see S.I. for additional pictures). c) The single residual liver schizont detected in a chimpanzee Dirk (day five post-challenge) appeared infiltrated by lymphomononuclear cells, as compared in d) to one of the numerous healthy schizonts observed in the control chimpanzee Peer (total of ca 2500 schizonts/300 liver sections, Giemsa-collophonium staining[28]) (see text). Bars correspond to 5 μm in panel a) and 20 μm in panels b) to d).

[0078] Figure 4: Blood parasitaemia courses in Groups II and III. a) chimpanzees from Group II and b-c) animals in Group III, following high dose (HD) or low dose (LD) challenges with NF54 sporozoites. Names of totally or partially protected animals are in bold. Hatched patterns correspond to control chimpanzees. Parasitaemia scales are different for each challenge, as expected from challenges with different numbers of sporozoites. Note that the day of patency in control and non-protected animals was the same for a given challenge inoculum within each group (in the above and in other groups not shown here).

Example 3

Sequence data and supplementary information

[0079] The following further information exemplifying the invention is supplied:

Sequence Data - Gene: full Sequence (K1 parasite)

- Protein: full Sequence (K1 parasite)
- Clones DG729 / DG679 (T9/96 parasite)
- Note on LSA-3 sequence in parasite 3D7

Gene & Protein - Structure . Restriction map . Hydrophobicity

- Oligonucleotides employed
- Organisation

Regions & Comments - NR-A . R1 . R2 . NR-B . R3 . NR-C
Conservation - of the gene

- of the sequence
- of repeat region R2
- comparaison of immunising and challenging sequences

Stage Specificity & Subcellular Location

Homologies - Intraspecies

[0080]

- Interspecies

Synthetic Peptides & Recombinant Proteins used for Chimpanzee Immunisations

[0081]

- Peptides CT1 . NR1 . NR2 . RE
- Recombinant proteins β-729. GST-729. GST-NN. GST-PC

Methods

References to Example 3

[0082]

SEQUENCE DATA

K1 PARASITE STRAIN: clone k1.2 Accession Nber AJ007010

GENE full sequence

```
        |  10      |  20      |  30      |  40      |  50       |  60      |  70      |  80      |  90      | 100
   1 atgacaaata gtaattacaa atcaaataat aaaacatata atgaaaataa taatgaacaa ataactacca tatttaatag aacaaatatg aatccgataa
 101 aaaaatgtca tatgagagaa aaaataaata agtacttttt tttgatcaaa atttgacat gcaccatttt aatatgggct gtacaatatg ataataacgt
 201 aagataaaaa actaaataat aaatataaat aaaaaaaaaa aaaaaaaaaa aaaaatcaac tatatagtat gtataatata tatatatata tatatatata
 301 tatatatata tatatattta tttttattta tttattaatt tttttttttt tatattatct ttttagtctg atataaacaa gagttggaaa aaaaatacgt
 401 atgtagataa gaaattgaat aaactattta acagaagttt aggagaatct caagtaaatg gtgaattagc tagtgaagaa gtaaaggaaa aaattcttga
 501 cttattagaa gaaggaaata cattaactga aagtgtagat gataataaaa atttagaaga agccgaagat ataaaggaaa atatcttatt aagtaatata
 601 gaagaaccaa aagaaaatat tattgacaat ttattaaata atattggaca aaattcagaa aaacaagaaa gtgtatcaga aaatgtacaa gtcagtgatg
 701 aacttttaa tgaattatta aatagtgtag atgttaatgg agaagtaaaa gaaaatattt tggaggaaag tcaagttaat gatgatattt ttaatagttt
 801 agtaaaaagt gttcaacaag aacaacaaca caatgttgaa gaaaaagttg aagaaagtgt agaagaaaat gacgaagaaa gtgtagaaga aaatgtagaa
 901 gaaaatgtag aagaaaatga cgacggaagt gtagcctcaa gtgttgaaga aagtatagct tcaagtgttg atgaaagtat agattcaagt attgaagaaa
1001 atgtagctcc aactgttgaa gaaatcgtag ctccaagtgt tgtagaaagt gtggctccaa gtgttgaaga aagtgtagaa gaaaatgttg aagaaagtgt
1101 agctgaaaat gttgaagaaa gtgtagctga aaatgttgaa gaaagtgtag ctgaaaatgt tgaagaaagt gtagctgaaa atgttgaaga aatcgtagct
1201 ccaactgttg aagaaatcgt agctccaact gttgaagaaa ttgtagctcc aagtgttgta gaaagtgtgg ctccaagtgt tgaagaaagt gtagaagaaa
1301 atgttgaaga aagtgtagct gaaaatgttg aagaaagtgt agctgaaaat gttgaagaaa gtgtagctga aaatgttgaa gaaagtgtag ctgaaaatgt
1401 tgaagaaagt gtagctgaaa atgttgaaga aatcgtagct ccaactgttg aagaaatcgt agctccaact gttgaagaaa ttgtagctcc aagtgttgta
1501 gaaagtgtgg ctccaagtgt tgaagaaagt gtagaagaaa atgttgaaga aagtgtagct gaaaatgttg aagaaagtgt agctgaaaat gttgaagaaa
1601 gtgtagctga aaatgttgaa gaaagtgtag ctgaaaatgt tgaagaaagt gtagctgaaa atgttgaaga aagtctagct gaaaatgttg aagaaagtgt
1701 agctgaaaat gttgaagaaa tcgtagctcc aactgttgaa gaaatcgtag ctccaactgt tgaagaaatt gtagctccaa gtgttgtaga aagtgtggct
1801 ccaagtgttg aagaaagtgt agaagaaaat gttgaagaaa gtgtagctga aaatgttgaa gaaagtgtag ctgaaaatgt tgaagaaagt gtagctgaaa
1901 atgttgaaga aagtgtagct gaaaatgttg aagaaatcgt agctccaact gttgaagaaa tcgtagctcc aactgttgaa gaaattgtag ctccaagtgt
2001 tgtagaaagt gtggctccaa gtgttgaaga aagtgtagaa gaaaatgttg aagaaagtgt agctgaaaat gttgaagaaa gtgtagctga aaatgttgaa
2101 gaaagtgtag ctgaaaatgt tgaagaaatc gtagctccaa ctgttgaaga aatcgtagct ccaactgttg aagaaattgt agctccaagt gttgtagaaa
2201 gtgtggctcc aagtgttgaa gaaagtgtag aagaaaatgt tgaagaaagt gtagctgaaa atgttgaaga aagtgtagct gaaaatgttg aagaaagtgt
2301 agctgaaaat gttgaagaaa gtgtagctga aaatgttgaa gaaatcgtag ctccaactgt tgaagaaatc gtagctccaa ctgttgaaga aattgtagct
2401 ccaagtgttg tagaaagtgt ggctccaagt gttgaagaaa gtgtagaaga aaatgttgaa gaaagtgtag ctgaaaatgt tgaagaaagt gtagctgaaa
2501 atgttgaaga aagtgtagct gaaaatgttg aagaaagtgt agctccaact gttgaagaaa ttgtagctcc aagtgttgaa gaaagtgtag ctccaagtgt
2601 tgaagaaagt gttgctgaaa acgttgcaac aaatttatca gacaatcttt taagtaattt attaggtggt atcgaaactg aggaaataaa ggacagtata
2701 ttaaatgaga tagaagaagt aaaagaaaat gtagtcacca caatactaga aaacgtagaa gaaactacag ctgaaagtgt aactactttt agtaacatat
2801 tagaggagat acaagaaaat actattacta atgatactat agaggaaaaa ttagaagaac tccacgaaaa tgtattaagt gccgctttag aaaataccca
2901 aagtgaagag gaaaagaaag aagtaataga tgtaattgaa gaagtaaaag aagaggtcgc taccacttta atagaaactg tggaacaggc agaagaaaag
3001 agcgcaaata caattacgga aatatttgaa aatttagaag aaaatgcagt agaaagtaat gaaaatgttg cagagaattt agagaaatta aacgaaactg
3101 tatttaatac tgtattagat aaagtagagg aaacagtaga aattagcgga gaaagtttag aaaacaatga aatggataaa gcatttttta gtgaaatatt
3201 tgataatgta aaaggaatac aagaaaattt attaacaggt atgtttcgaa gtatagaaac cagtatagta atccaatcag aagaaaaggt tgatttgaat
3301 gaaaatgtgg ttagttcgat tttagataat atagaaaata tgaaagaagg tttattaaat aaattagaaa atatttcaag tactgaaggt gttcaagaaa
3401 ctgtaactga acatgtagaa caaaatgtat atgtggatgt tgatgttcct gctatgaaag atcaattttt aggaatatta aatgaggcag gagggttgaa
3501 agaaatgttt tttaatttgg aagatgtatt taaaagtgaa agtgatgtaa ttactgtaga agaaattaag gatgaaccgg ttcaaaaaga ggtagaaaaa
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3701 aatccataga aatatcttca gattctaaag aagaaactga atctattaaa gataaagaaa aagatgtttc actagttgtt gaagaagttc aagacaatga
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4001 taatagatgc aaaagatgat acattagaaa aagttattga agaggaacat gatataacga cgacgttgga tgaagttgta gaattaaaag atgtcgaaga
4101 agacaagatc gaaaaagtat ctgatttaaa agatcttgaa gaagatatat taaaagaagt aaaagaaatc aaagaacttg aaagtgaaat tttagaagat
4201 tataaagaat taaaaactat tgaaacagat attttagaag agaaaaaaga aatagaaaaa gatcattttg aaaaattcga agaagaagct gaagaaataa
4301 aagatcttga agcagatata ttaaaagaag tatcttcatt agaagttgaa gaagaaaaaa aattagaaga agtacacgaa ttaaaagaag aggtagaaca
4401 tataataagt ggtgatgcgc atataaaagg tttggaagaa gatgatttag aagaagtaga tgatttaaaa ggaagtatat tagacatgtt aaagggagat
4501 atggaattag gggatatgga taaggaaagt ttagaagatg taacaacaaa acttggagaa agagttgaat ccttaaaaga tgttttatct agtgcattag
4601 gcatggatga agaacaaatg aaaacaagaa aaaaagctca aagacctaag ttggaagaag tattattaaa agaagaggtt aaagaagaac caaagaaaaa
4701 aataacaaaa aagaaagtaa ggtttgatat taaggataag gaaccaaaag atgaaatagt agaagttgaa atgaaagatg aagatataga agaagatgta
4801 gaagaagata tagaagaaga tatagaagaa gataaagttg aagatataga tgaagatata gatgaagata taggtgaaga caaagatgaa gttatagatt
4901 taatagtcca aaaagagaaa cgcattgaaa aggttaaagc gaaaaagaaa aaattagaaa aaaaagttga agaaggtgtt agtggtctta aaaaacacgt
5001 agacgaagta atgaaatatg ttcaaaaaat tgataaagaa gttgataaag aagtatctaa agctttagaa tcaaaaaatg atgttactaa tgttttaaaa
5101 caaaatcaag atttttttag taaagttaaa aacttcgtaa aaaaatataa agtatttgct gcaccattca tatctgccgt tgcagcattt gcatcatatg
5201 tagttgggtt ctttacattt tcttttattt catcatgtgt aacaatagct tcttcaactt acttattatc aaaagttgac aaaactataa ataaaaataa
5301 ggagagaccg ttttattcat ttgtatttga tatctttaag aatttaaaac attatttaca acaaatgaaa gaaaaattta gtaaagaaaa aaataataat
5401 gtaatagaag taacaaacaa agctgagaaa aaaggtaatg tacaggtaac aaataaaacc gagaaaacaa ctaaagttga taaaaataat aaagtaccga
5501 aaaaaagaag aacgcaaaaa tcaaaataa . 5529
        |  10      |  20      |  30      |  40      |  50       |  60      |  70      |  80      |  90      | 100
```

Complete nucleotide sequence of the 5529 base-pair (bp) *lsa-3* gene. Bolded is a 168 bp intron; underlined are the 3 repeat regions R1, R2 and R3.

[illegible] sequence:

```
   1 MTNSNYKSNN KTYNENNNEQ ITTIFNRTNM NPIKKCHMRE KINKYFFLIK ILTCTILIWA VQYDNNSDIN KSWKKNTYVD
  81 KKLNKLFNRS LGESQVNGEL ASEEVKEKIL DLLEEGNTLT ESVDENKNLE EAEDIKENIL LSNIEEPKEN IIDNLLNNIG
 161 QNSEKQESVS ENVQVSDELF NELLNSVDVN GEVKENILEE SQVNDDIFNS LVKSVQQEQQ HNVEEKVEES VEENDEESVE
 241 ENVEENVEEN DDGSVASSVE ESIASSVDES IDSSIEENVA PTVEEIVAPS VVESVAPSVE ESVEENVEES VAENVEESVA
 321 ENVEESVAEN VEESVAENVE EIVAPTVEEI VAPTVEEIVA PSVVESVAPS VEESVEENVE ESVAENVEES VAENVEESVA
 401 ENVEESVAEN VEESVAENVE EIVAPTVEEI VAPTVEEIVA PSVVESVAPS VEESVEENVE ESVAENVEES VAENVEESVA
 481 ENVEESVAEN VEESVAENVE ESVAENVEES VAENVEEIVA PTVEEIVAPT VEEIVAPSVV ESVAPSVEES VEENVEESVA
 561 ENVEESVAEN VEESVAENVE ESVAENVEEI VAPTVEEIVA PTVEEIVAPS VVESVAPSVE ESVEENVEES VAENVEESVA
 641 ENVEESVAEN VEEIVAPTVE EIVAPTVEEI VAPSVVESVA PSVEESVEEN VEESVAENVE ESVAENVEES VAENVEESVA
 721 ENVEEIVAPT VEEIVAPTVE EIVAPSVVES VAPSVEESVE ENVEESVAEN VEESVAENVE ESVAENVEES VAPTVEEIVA
 801 PSVEESVAPS VEESVAENVA TNLSDNLLSN LLGGIETEEI KDSILNEIEE VKENVVTTIL ENVEETTAES VTTFSNILEE
 881 IQENTITNDT IEEKLEELHE NVLSAALENT QSEEEKKEVI DVIEEVKEEV ATTLIETVEQ AEEKSANTIT EIFENLEENA
 961 VESNENVAEN LEKLNETVFN TVLDKVEETV EISGESLENN EMDKAFFSEI FDNVKGIQEN LLTGMFRSIE TSIVIQSEEK
1041 VDLNENVVSS ILDNIENMKE GLLNKLENIS STEGVQETVT EHVEQNVYVD VDVPAMKDQF LGILNEAGGL KEMFFNLEDV
1121 FKSESDVITV EEIKDEPVQK EVEKETVSII EEMEENIVDV LEEEKEDLTD KMIDAVEESI EISSDSKEET ESIKDKEKDV
1201 SLVVEEVQDN DMDESVEKVL ELKNMEEELM KDAVEINDIT SKLIEETQEL NEVEADLIKD MEKLKELEKA LSEDSKEIID
1281 AKDDTLEKVI EEEHDITTTL DEVVELKDVE EDKIEKVSDL KDLEEDILKE VKEIKELESE ILEDYKELKT IETDILEEKK
1361 EIEKDHFEKF EEEAEEIKDL EADILKEVSS LEVEEEKKLE EVHELKEEVE HIISGDAHIK GLEEDDLEEV DDLKGSILDM
1441 LKGDMELGDM DKESLEDVTT KLGERVESLK DVLSSALGMD EEQMKTRKKA QRPKLEEVLL KEEVKEEPKK KITKKKVRFD
1521 IKDKEPKDEI VEVEMKDEDI EEDVEEDIEE DIEEDKVEDI DEDIDEDIGE DKDEVIDLIV QKEKRIEKVK AKKKKLEKKV
1601 EEGVSGLKKH VDEVMKYVQK IDKEVDKEVS KALESKNDVT NVLKQNQDFF SKVKNFVKKY KVFAAPFISA VAAFASYVVG
1681 FFTFSLFSSC VTIASSTYLL SKVDKTINKN KERPFYSFVF DIFKNLKHYL QQMKEKFSKE KNNNVIEVTN KAEKKGNVQV
1761 TNKTEKTTKV DKNNKVPKKR RTQKSKZ 1786
```

Complete peptide sequence of the 1786 amino-acid (aa) LSA-3 protein. Bolded are 3 potential start sites; underlined are the 3 repeat regions R1, R2 and R3.

T9/96 PARASITE CLONE — **Accession Nber AJ007011**

Nucleotidic sequence:

```
   1' agtgatgaac tttttaatga attattaaat agtgtagatg ttaatggaga agtaaaagaa aatatttgg aggaaagtca
  81' agttaatgac gatatttta atagtttagt aaaaagtgtt caacaagaac aacaacacaa tgttgaagaa aaagttgaag
 161' aaagtgtaga agaaaatgac gaagaaagtg tagaagaaaa tgtagaagaa aatgtagaag aaaatgacga cggaagtgta
 241' gcctcaagtg ttgaagaaag tatagcttca agtgttgatg aaagtataga ttcaagtatt gaagaaaatg tagctccaac
 321' tgttgaagaa atcgtagctc caactgttga agaaattgta gctccaagtg ttgtagaaag tgtggctcca agtgttgaag
 401' aaagtgtagc tccaagtgtt gaagaaagtg tagctgaaaa tgttgaagaa agtgtagctg aaaatgttga agaaatcgta
 481' gctccaagtg ttgaagaaag tgtagctgaa aatgttgaag aaagtgtagc tgaaaatgtt gaagaaagtg tagctgaaaa
 561' tgttgaagaa agtgtagctg aaaatgttga agaaagtgta gctgaaaatg ttgaagaaat cgtagctcca actgttgaag
 641' aaagtgtagc tccaactgtt gaagaaattg tagctccaac tgttgaagaa agtgtagctc caactgttga agaaattgta
 721' gttccaagtg ttgaagaaag tgtagctcca agtgttgaag aaagtgtagc tgaaaatgtt gaagaaagtg tagctgaaaa
 801' tgttgaagaa agtgtagctg aaaatgttga agaaagtgta gctgaaaatg ttgaagaaag tgtagctgaa aatgttgaag
 881' aaatcgtagc tccaagtgtt gaagaaatcg tagctccaac tgttgaagaa agtgttgctg aaaacgttgc aacaaatttа
 961' tcagacaatc ttttaagtaa tttattaggt ggtatcgaaa ctgaggaaat aaaggacagt atattaaatg agatagaaga
1041' agtaaaagaa aatgtagtca ccacaatact agaaaaagta gaagaaacta cagctgaaag tgtaactact tttagtaata
1121' tattagagga gatacaagaa aatactatta ctaatgatac tatagaggaa aaattagaag aactccacga aaatgtatta
1201' agtgccgctt tagaaaatac ccaaagtgaa gaggaaaaga aagaagtaat agatgtaatt gaagaagtaa aagaagaggt
1281' cgctaccact ttaatagaaa ctgtggaaca ggcagaagaa gagagcgaaa gtacaattac ggaaatattt gaaaatttag
1361' aagaaaatgc agtagaaagt aatgaaaaag ttgcagagaa tttagagaaa ttaaacgaaa ctgtatttaa tactgtatta
1441' gataaagtag aggaaacagt agaaattagc ggagaaagtt tagaaaacaa tgaaatggat aaagcatttt ttagtgaaat
1521' atttgataat gtaaaaggaa tacaagaaaa tttattaaca ggtatgtttc gaagtataga aaccagtata gtaatccaat
1601' cagaagaaaa ggttgatttg aatgaaaatg tggttagttc gattttagat aatatagaaa atatgaaaga aggtttatta
1681' aataaattag aaaatatttc aagtactgaa gg 1712'
```

Partial nucleotide sequence of the *lsa-3* gene in the Thai parasite clone T9/96. Bolded is the sequence of insert DG729. Insert DG679, the largest among the LSA-3 insert family (see text of the present article and Guérin-Marchand *et al.*, 1987), spans from nucl. 32' to nucl. 1712'. Underlined are the adjacent repeat regions R1 and R2. Position 1' corresponds to nucl. 694 in the original K1 sequence.

Peptide sequence

```
   1' SDELPNELLN SVDVNGEVKE NILEESQVND DIFNSLVKSV QQEQQHNVEE KVEESVEEND EESVEENVEE NVERNDDGSV
  81' ASSVEESIAS SVDESIDSSI EENVAPTVEE IVAPTVEEIV APSVVESVAP SVEESVAPSV EESVAENVEE SVAENVEEIV
 161' APSVEESVAE NVEESVAENV EESVAENVEE SVAENVEESV AENVEEIVAP TVEESVAPTV EEIVAPTVEE SVAPTVEEIV
 241' VPSVEESVAP SVEESVAENV EESVAENVEE SVAENVEESV AENVEESVAE NVEEIVAPSV EEIVAPTVEE SVAENVATNL
 321' SDNLLSNLLG GIETEEIKDS ILNEIEEVKE NVVTTILEKV EETTAESVTT FSNILEEIQE NTITNDTIEE KLEELHENVL
 401' SAALENTQSE EEKKEVIDVI EEVKEEVATT LIETVEQAEE ESESTITEIF ENLEENAVES NEKVAENLEK LNETVFNTVL
 481' DKVEETVEIS GESLENNEMD KAFFSEIFDN VKGIQENLLT GMFRSIETSI VIQSEEKVDL NENVVSSILD NIENMKEGLL
 561' NKLENISSTE 570'
```

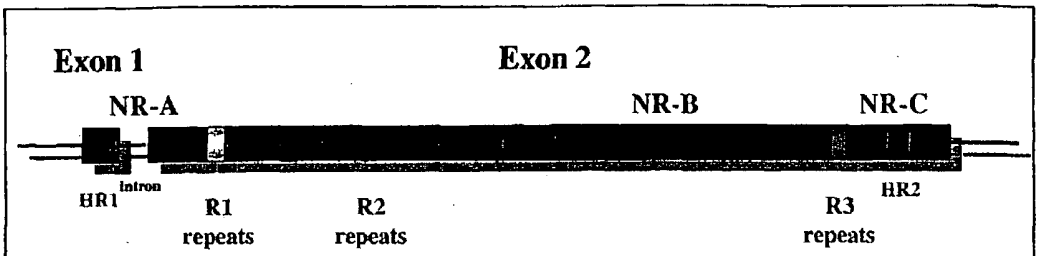
Partial peptide sequence of the LSA-3 protein in the Thai parasite clone T9/96. Bolded is the sequence of insert DG729. Insert DG679, the largest among the LSA-3 insert family (see text of the present article and Guérin-Marchand *et al.*, 1987), spans from aa 12' to aa 570'. Underlined are the 2 adjacent repeat regions R1 and R2. Position 1' corresponds to aa 176 in the original K1 sequence.

Note on LSA-3 sequence in parasite 3D7

The *lsa-3* gene sequence in parasite clone 3D7 (derived from strain NF54 used in the present article for chimpanzee challenges) is found in the complete sequence of *P. falciparum* Chromosome 2 (Gardner *et al.*, 1998) where it was annotated as *resa-h3* (Accession Number AE001424).

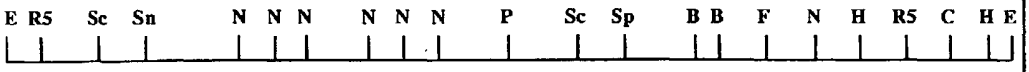
LSA-3 GENE & PROTEIN

K1 Parasite Strain - clone k1.2

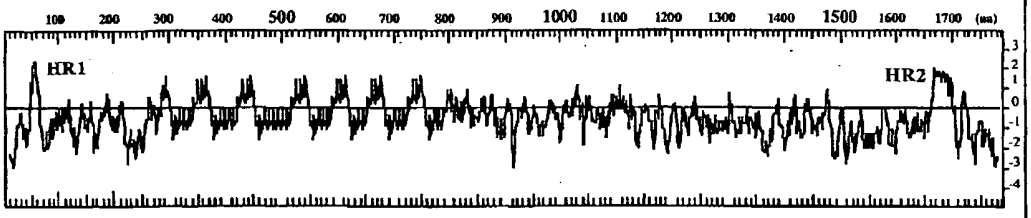


Restriction map

B: Bgl II C: Cla I E: Eco RI F: Fsp I H: Hind III
N: Nla IV P: Pvu II R5: Eco RV Sc: Sca I Sn: Sna BI Sp: Spe I
1 Kb



Hydrophobicity - HYDROPHOBIC PLOT (KYTE & DOOLITTLE)



Oligonucleotides employed

Name	Location	Utilisation
i1(+) / i2(-)	nucl. 161-184 / 454-432	intron amplification, RT-PCR
CTL1(+) / CTL2(-)	nucl. 649-677 / 942-919	amplification of the NR2 peptide-coding region
S1(+) / S2(-)	nucl. 695-722 / 824-799	*lsa-3* gene detection in *P. falciparum*

Gene [5529 bp]

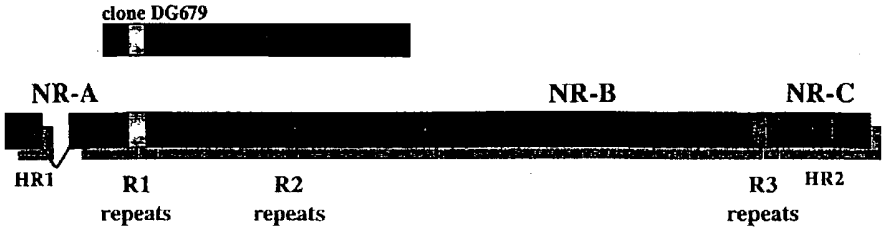
Regions	Length	Location	Regions	Length	Location
NR-A	834 bp	nucl. 1-834	R2 repeats	1623 bp	nucl. 1003-2625
Exon 1	198 bp	nucl. 1-198	NR-B	2148 bp	nucl. 2626-4773
Intron	168 bp	nucl. 199-366	R3 repeats	126 bp	nucl. 4774-4899
Exon 2	5164 bp	nucl. 367-5529	NR-C	630 bp	nucl. 4900-5529
R1 repeats	168 bp	nucl. 835-1002			

Protein [786 amino acids - Predicted MW : 200 kDa]

Regions	Length	Location	
NR-A	278 aa	aa 1-278	Non-repeated region A
HR1	18 aa	aa 46-63	Hydrophobic region 1: putative signal peptide
R1	56 aa	aa 223-278	Conserved repeat region
R2	541 aa	aa 279-819	Polymorphic repeat region
NR-B	716 aa	aa 820-1535	Non-repeated region B
R3	42 aa	aa 1536-1577	Conserved repeat region
NR-C	210 aa	aa 1578-1786	Non-repeated region C
HR2	33 aa	aa 1662-1694	Hydrophobic region 2: putative transmembrane domain

REGIONS & COMMENTS

k1.2 and T9/96 clones



NR-A

```
  1 MTNSNYKSNN KTYNENNNEQ ITTIFNRTNM NPIKKCHMRE KINKYFFLIK ILTCTILIWA VQYDNNSDIN
 71 KSWKKNTYVD KKLNKLFNRS LGESQVNGEL ASEEVKEKIL DLLEEGNTLT ESVDDNKNLE EAEDIKENIL
141 LSNIEEPKEN IIDNLLNNIG QNSEKQESVS ENVQVSDELF NELLNSVDVN GEVKENILEE SQVNDDIFNS
211 LVKSVQQEQQ HN 222
```

Underlined and bolded are the 3 potential start sites; in green is a stretch of 17 uncharged and hydrophobic residues (HR1), preceeded and followed by two short positively charged regions. As confirmed by the combined neural approach documented in Nielsen *et al.* (1997): 1) this constitutes a potential signal sequence peptide, consistent with the subcellular location of LSA-3 in sporozoites and in liver forms, 2) most likely cleavage site is located between aa 63 and 64. Underlined is the NR2 peptide-coding region which shows a perfect conservation among *P. falciparum* parasites.

R1

```
223 VEEK VEES VEEN DEES VEEN VEEN VEEN DDGS VASS VEES IASS VDES IDSS IEEN 278
```

R1 is distinguished from region R2 by its specific tetrapeptide motifs and an extremely high conservation in T9/96 (100% at both nucleotidic and peptidic levels) and 3D7 (1 point mutation over 168bp/56aa, see sequence AE001424 in Gardner *et al.*, 1998) parasite clones.

R2 / k1.2 clone

```
279 VAPT  VEEIVAPS  VVESVAPS  VEESVEEN
307 VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN

339 VEEIVAPT  VEEIVAPT  VEEIVAPS  VVESVAPS  VEESVEEN
379 VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN

419 VEEIVAPT  VEEIVAPT  VEEIVAPS  VVESVAPS  VEESVEEN
459 VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN

515 VEEIVAPT  VEEIVAPT  VEEIVAPS  VVESVAPS  VEESVEEN
555 VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN

587 VEEIVAPT  VEEIVAPT  VEEIVAPS  VVESVAPS  VEESVEEN
627 VEESVAEN  VEESVAEN  VEESVAEN

651 VEEIVAPT  VEEIVAPT  VEEIVAPS  VVESVAPS  VEESVEEN
691 VEESVAEN  VEESVAEN  VEESVAEN  VEESVAEN

723 VEEIVAPT  VEEIVAPT  VEEIVAPS  VVESVAPS  VEESVEEN
763 VEESVAEN  VEESVAEN  VEESVAEN

787 VEESVAPT  VEEIVAPS  VEESVAPS
811 VEESVAEN 818
```

R2 / T9/96 clone

104' VAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVAPS
140' **VEESVAEN VEESVAEN**
156' VEEIVAPS
164' **VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN**
204' VEEIVAPT VEESVAPT VEEIVAPT VEESVAPT VEEIVVPS VEESVAPS
252' **VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN**
292' VEEIVAPS VEEIVAPT
308' **VEESVAEN 315'**

Bolded are stretches of tandemly repeated and conserved octapeptides VEESVAEN which can vary in number, from 2 to 7 in both strains. Underlined are the highly conserved 40 aa repeated blocks which separate these stretches in strain K1. In clone T9/96 , no particular organization is observed in R2. This region is nevertheless composed of similar and conserved tetrapeptides compared to strain K1, except one variant VVPS which is specific for T9/96.

NR-B

819 VA TNLSDNLLSN LLGGIETEEI KDSILNEIEE VKENVVTTIL ENVEETTAES VTTFSNILEE
881 IQENTITNDT IEEKLEELHE NVLSAALENT QSEEEKKEVI DVIEEVKEEV ATTLIETVEQ AEEKSAETIT
951 EIFENLEENA VESNENVAEN LEKLNETVFN TVLDKVEETV EISGESLENN EMDKAFFSEI FDNVKGIQEN
1021 LLTGMFRSIE TSIVIQSEEK VDLNENVVSS ILDNIENMKE GLLNKLENIS STEGVQETVT EHVEQNVYVD
1091 VDVPAMKDQF LGILNEAGGL KEMFFNLEDV FKSESDVITV EEIKDEPVQK EVEKETVSII EEMEENIVDV
1161 LEEEKEDLTD KMIDAVEESI EISSDSKEET ESIKDKEKDV SLVVEEVQDN DMDESVEKVL ELKNMEEELM
1231 KDAVEINDIT SKLIEETQEL NEVEADLIKD MEKLKELEKA LSEDSKEIID AKDDTLEKVI EEEHDITTTL
1301 DEVVELKDVE EDKIEKVSDL KDLEEDILKE VKEIKELESE ILEDYKELKT IETDILEEKK EIEKDHFEKF
1371 EEEAEEIKDL EADILKEVSS LEVEEEKKLE EVHELKEEVE HIISGDAHIK GLEEDDLEEV DDLKGSILDM
1441 LKGDMELGDM DKESLEDVTT KLGERVESLK DVLSSALGMD EEQMKTRKKA QRPKLEEVLL KEEVKEEPKK
1511 KITKKKVRFD IKDK[redacted]EM 1535

Underlined is the partial NR-B region of insert DG679 (parasite clone T9/96) which shows a high degree of conservation with K1 sequences and contains only 6 bp substitutions leading to 5 aa mutations (bolded). Shaded is the highly conserved HLA-B53 restricted epitope la90 identified by Aidoo *et al.* (2000).

R3

1536 KDED IEED VEED IEED IEED KVED IDED IDED IGED KDEV ID 1577

The same regular spacing of the hydrophobic isoleucine and valine residues is observed in region R3 which is predicted, according to its HCP analysis (not shown), to adopt an α-helical conformation and is preceded by a cluster of helix-breakers (proline) alternating with ß-sheet segments. This region also shows a high degree of conservation with LSA-3 sequences in clone 3D7 (see sequence AE001424 in Gardner *et al.*, 1998) and in isolates from various geographical origins (Daubersies, P. *et al.*, in preparation).

NR-C

1578 LIV QKEKRIEKVK AKKKKLEKKV EEGVSGLKKH VDEVMKYVQK IDKEVDKEVS KALESKNDVT
1641 NVLKQNQDFF SKVKNFVKKY **KVFAAPFISA VAAFASYVVG FFTFSLFSSC VTIASSTYLL** SKVDKTINKN
1711 KERPFYSFVF DIFKNLKHYL QQMKEKFSKE KNNNVIEVTN KAEKKGNVQV TNKTEKTTKV DKNNKVPKKR
1781 RTQKSKZ 1786

Bolded (and in green) is a second hydrophobic region (HR2) which could constitute a transmembrane domain, consistent with the subcellular location of the antigen in sporozoites and in liver forms.

CONSERVATION

Conservation of the gene

LSA-3 gene and protein detected in 100 % of _P. falciparum_ parasites by:

• **PCR ANALYSIS** performed with S1(+) / S2(-) primers on:

- 70 isolates from Ivory Coast, Madagascar, Myanmar, Brazil and Columbia
- 12 Thai sporozoite strains
- 6 laboratory strains or clones (K1, T9/96, NF54, Palo Alto, 150, 3D7)
- 23 Senegalese isolates - Data published in Bottius *et al.* (1996) [where clone DG157 is a member of the LSA-3 clone family and encodes for a part of region NR-A]

The expected 130 bp amplification product was found in the 111 samples

•**IFAT** performed with anti-NR2 peptide and anti-GST-PC antibodies (mouse and chimpanzee sera) on:

- 30 Thai sporozoite strains
- 2 infected liver sections: one from a *Cebus* (day 5 post-challenge) and one from a chimpanzee (day 6 post-challenge)

Detection in the 32 samples
100 % of positive parasites in each assay

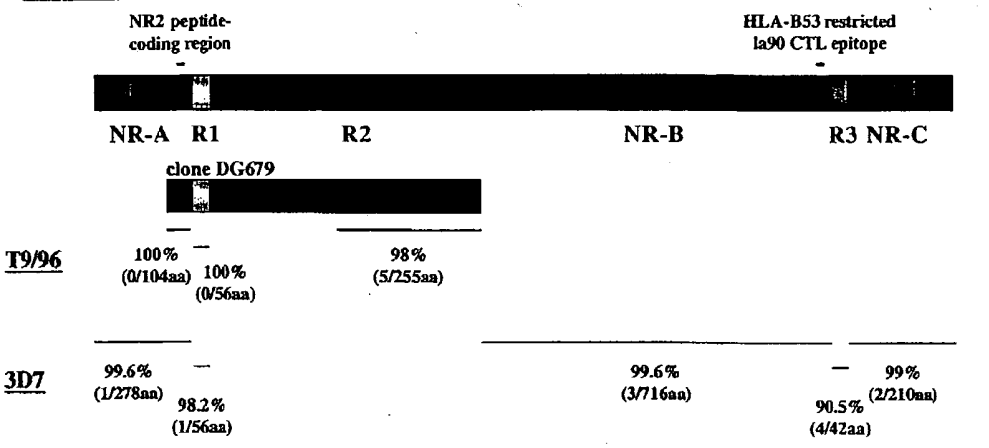
Conservation of the sequence

Data published in Ben Mohamed *et al.* (1997)
Direct PCR sequencing performed with CTL1(+) / CTL2(-) primers from nucl. 740 to nucl. 861 (122 bp) on:
. 5 strains or clones (K1, T9/96, NF54, Palo Alto, 3D7)
. 7 African, 5 Brazilian, 3 Malagasi, 3 Burmese isolates
. 5 Thai clones (Druilhe et al., 1998)

100 % bp conservation in 28 samples for:

Data published in Aidoo *et al.* (2000)
From nucl. 4741 to nucl. 4767 (27 bp):
1 silent mutation in 12/18 Gambian isolates
[nucl. 4746/codon 1526: cca -> ccc]

97.5 % conservation in nucleotides
100 % conservation in amino acids for:



Homology at amino acid level (nber of mutation(s)/length of the region analysed) in parasite clones T9/96 and 3D7

Precise position and description of bp/aa mutations in parasite K1, T9/96 and 3D7 is detailed in 2 tables from section "comparaison of immunising and challenging sequences". Conservation of the polymorphic repeat region R2 is analysed in the following section.

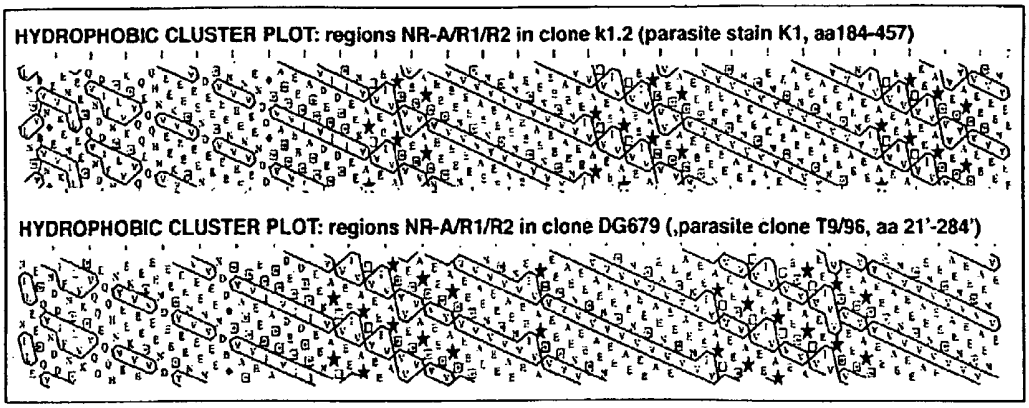
Conservation of the repeat region R2

Conservation of R2 motif sequences

MOTIFS		*P. FALCIPARUM* LINES		
PEPTIDIC	NUCLEOTIDIC	K1	T9/96	3D7
VAEN	gta gct gaa aat --t --- --- --c	30/31 1/31	12/13 1/13	9/10 1/10
VAPS	gta gct cca agt --g --- --- ---	9/16 7/16	5/6 1/6	16/17 1/17
VAPT	gta gct cca act --- --- --- --a	14/14 -	7/7 -	7/9 2/9
VEES	gtt gaa gaa agt	42/42	17/17	15/15
VEEI	gtt gaa gaa atc --- --- --- --t	13/20 7/20	5/8 3/8	16/22 6/22
VEEN	gta gaa gaa aat	11/11	-	1/1
VVES	gtt gta gaa agt --c --- --- ---	7/7 -	- -	- 1/1
VVPS	gta gtt cca agt	-	1/1	-
VVPT	gta gtt cca act	-	-	2/2

Peptide and nucleotide sequence comparison of R2 tetrapeptidic motifs between K1, T9/96 and 3D7 parasites. Although the organization of these tetrapeptide motifs varies within R2 (see section "regions & comments" for K1, and T9/96 and see sequence AE001424 in Gardner *et al.* (1998) for 3D7), conservation of their sequences remains extremly high (e.g. only 3 strain specific tetrapeptides (VVPS, VVPT) among a total of 231 motifs and no single nucleotide mutation in the 74 VEES, 21 VAPT, 12 VEEN motifs.

Conservation of R2 helicity



Prediction of LSA-3 conformation (K1 strain) by hydrophobic cluster plot (HCP) analysis (for symbols, see Gaboriaud *et al.*, 1987) reveals a regular organization of the R1-R2 repeat regions, in a succession of α-helical stretches interrupted by the helix-breaker proline residues (tetrapeptides VAPT). This α-helical conformation is also strongly suggested by the remarkable regular spacing, every 4 residues, of the hydrophobic valine throughout the entire R1-R2 block, i.e. 597 aa. To a lesser extend, the same regular spacing of the hydrophobic isoleucine and valine residues is observed in the R3 repeat region which is predicted, according to its HCP (not shown), to adopt an α-helical conformation and is preceded by a cluster of helix-breakers (proline) alternating with ß-sheet segments.

Regions R1-R2 from T9/96 shows a different organization since sequences separating the stretches of tandemly repeated octapeptide VEESVAEN consist of a mosaic of various tetrapeptides also found in blocks R1-R2 of clone k1.2. Nevertheless and according to its HCP, the secondary structure of R1-R2 seems perfectly conserved in T9/96 compared to K1, with the same succession of α-helical stretches interrupted by the proline helix-breaker residues. This result is strongly suggestive of important structural constraints at least on this part of the protein.

Conservation of R2 conformation

Antibodies	recombinant proteins and peptides (ELISA)		NF54 sporozoites (IFAT)
	from K1	from T9/96	
anti-RE (T9/96)	+ / GST-NN	+ / GST-729	+
anti-GST-NN (K1)	+ / GST-NN	+ / RE	+

As shown in this table, conservation of R2 conformation is suggested by the constant recognition of recombinant proteins and peptides (K1 and T9/96 derived sequences) in ELISA and of NF54 sporozoites in IFAT by anti-RE (T9/96) or anti-GST-NN (K1) antibodies (mouse sera and human immunopurified antibodies).

Comparison between immunisations and challenging sequences

Mutations identified and localisation

LSA-3 Regions[1]	Clones[2]	Mutated nucleotide[3]	Mutated codon[3]	Original K1 sequence[4]	Mutated sequence[4]
NR-A (1-834)	3D7	191	64	gat (D)	gct (A)
R1 (835-1002)	3D7	926	253	gga (G)	gct (E)
NR-B (2626-4773)	T9/96	2754	862	aac (N)	aaa (K)
	T9/96	2796	876	aac (N)	aat *sil.*
	3D7 + T9/96	2998	944	aag (K)	gag (E)
	T9/96	3005	946	gca (A)	gag (E)
	3D7 + T9/96	3008	947	aat (N)	agt (S)
	T9/96	3066	966	aat (N)	aaa (K)
	3D7	3972	1268	gaa (E)	gag *sil.*
	3D7	4546	1460	aca (T)	gca (A)
	3D7	4650	1494	aag (K)	aaa *sil.*
R3 (4774-4899)	3D7	4791	1541	gaa (E)	gat (D)
	3D7	4798	1544	gta (V)	ata (I)
	3D7	4810	1548	ata (I)	gta (V)
	3D7	4870-71	1567-68	-	12 bp ins.[5]
NR-C (4900-5529)	3D7	4940	1591	gcg (A)	gag (E)
	3D7	5508	1780	aga (R)	agt (S)

Position in the reference *lsa-3* gene (strain K1) and description of the mutations identified in parasites clones T9/96 and 3D7 (which was originally cloned from strain NF54 and is considered here as representative of NF54 for complete comparison purposes). As reported in section "conservation of the sequence", NR2 peptide-coding region of the NF54 strain used for the chimpanzee challenges was found 100 % homologuous to K1 sequence.

1. Comments on region R2 from K1, T9/96 and 3D7 parasites are given in the preceeding section. Numbers in brackets define first and last nucleotides of the corresponding region in strain K1. 2. 3D7 sequences analysed here cover the entire gene and were defined by compiling data from 3 different sources: 1) construct VR2555 which contains a PCR-amplified truncated *lsa-3* gene (nucl. 432-5095; P. Daubersies, unpublished data), 2) construct VR2556 which contains a full-length PCR-amplified LSA-3 cDNA (Hoffman S., personal communication), 3) *lsa-3* gene sequence identified in *P. falciparum* Chromosome 2 (seq. AE001424 in Gardner *et al.*, 1998). Mutations were considered as such if they were observed in at least 2 out of 3 sequences. 3. Numbers for mutated nucleotides and codons correspond to their location in the reference *lsa-3* gene and protein respectively (in strain K1). 4. Original and mutated codons are followed in brackets with the corresponding amino acid (one-letter code). 5. 12 base pair insertion "gaagatatagat", leading to a 4 amino acid insertion "EDID".

Correspondences and homologies

LSA-3 regions		LSA-3 sequences[1]					
		in strain K1		in clone T9/96		in clone 3D7	
		sequenced	immunis.[2]	sequenced	immunis.[3]	sequenced[4]	challenge[5]
NR-A	length in base pairs	834	60 (CT1)	316	141 (GST-729)	834	60 + 141
	location in gene	1-834	586-645	519-834	694-834	1-834	586-645 + 694-834
	length in amino acids	278	20	104	47	278	20 + 47
	location in protein	1-278	140-159	119-222	176-222	1-278	140-159 + 176-222
	nucleotid. mutation(s)			0	0	1	0
	aa mutation(s)			0	0	1	0
R1	length in base pairs	168	-	168	168 (GST-729)	168	168
	location in gene	835-1002		835-1002	835-1002	835-1002	835-1002
	length in amino acids	56	-	56	56	56	56
	location in protein	223-278		223-278	223-278	223-278	223-278
	nucleotid. mutation(s)			0	0	1	1
	aa mutation(s)			0	0	1	1
R2[6]	length in base pairs	1623	240 (GST-NN)	636 (full seq.)	141 (GST-729)	924 (full seq.)	924
	location in gene	1003-2625	1269-1509				
	length in amino acids	541	80	212	47	308	308
	location in protein	279-819	369-448				
NR-B	length in base pairs	2148	2006 (GST-PC)	764	-	2148	2009
	location in gene	2626-4773	2769-4773	2626-3389		2626-4773	2769-4773
	length in amino acids	716	667	255	-	716	667
	location in protein	820-1535	869-1535	820-1074		820-1535	869-1535
	nucleotid. mutations)			6		5	5
	aa mutation(s)			5		3	3
R3	length in base pairs	126	126 (GST-PC)	-	-	126	126
	location in gene	4774-4899	4774-4899			4774-4899	4774-4899
	length in amino acids	42	42	-	-	42	42
	location in protein	1536-1577	1536-1577			1536-1577	1536-1577
	nucleotid. mutations					4	4
	aa mutation(s)					4	4
NR-C	length in base pairs	630	630 (GST-PC)	-	-	630	630
	location in gene	4900-5529	4900-5529			4900-5529	4900-5529
	length in amino acids	210	210	-	-	210	210
	location in protein	1578-1786	1578-1786			1578-1786	1578-1786
	nucleotid. mutations					2	2
	aa mutation(s)					2	2
Non-repeated regions (NR-A, -B, -C)	total length in bp/aa	3612/1204	2695/898	1080/360		3612/1204	2836/944
	total nber nucl./aa mut.			6/5		8/6	7/5
	nucl./aa homology (%)			**99.4/98.6**		**99.8/99.5**	**99.8/99.5**
Conserved regions (NR-A, -B, -C, R1,R3)	total length in bp/aa	3906/1302	2821/940	1248/416		3906/1302	3130/1042
	total nber nucl./aa mut.			6/5		13/11	12/10
	nucl./aa homology (%)			**99.5/98.8**		**99.7/99.1**	**99.6/99.0**

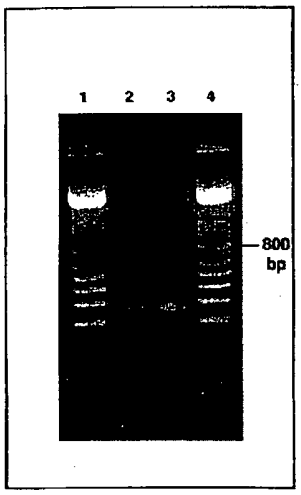
Definition and comparison of immunising and challenging sequences. As in the preceeding table, *lsa-3* sequence in clone 3D7 (originally cloned from NF54 strain) is considered here as representative of the actual NF54 strain used for sporozoite challenges.

1: All sequence locations (bp and aa) correspond to the reference numbering in *lsa-3* gene and protein from strain K1. **2:** Immunising sequences in strain K1 correspond to peptide CT1 and recombinant proteins GST-NN and GST-PC. **3:** Immunising sequences in clone T9/96 correspond to peptides NR1, NR2, and RE and recombinant protein GST-729 from which these 3 peptides were derived. **4:** See note (2) in the preceeding table. **5:** Challenging sequences are defined as 3D7 sequences corresponding to cumulated immunising sequences from both K1 and T9/96 parasites. **6:** A more detailed analysis of R2 is given in the preceeding section. Due to length polymorphism, numbering in region R2 is non-relevant in parasites other than K1. Lengths given for T9/96 and 3D7 correspond to their respective fully sequenced region R2.

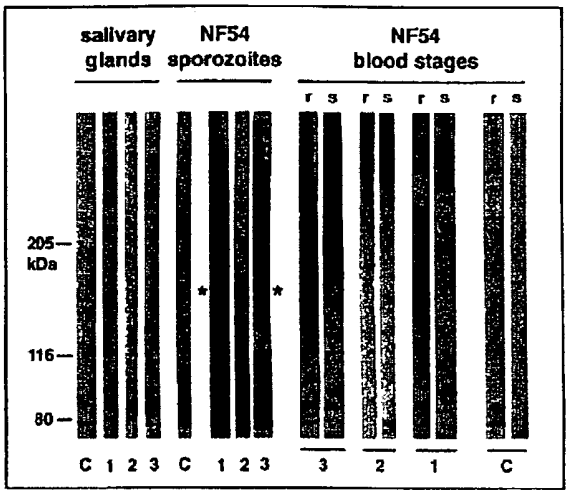
STAGE SPECIFICITY & SUBCELLULAR LOCATION

SPOROZOITES

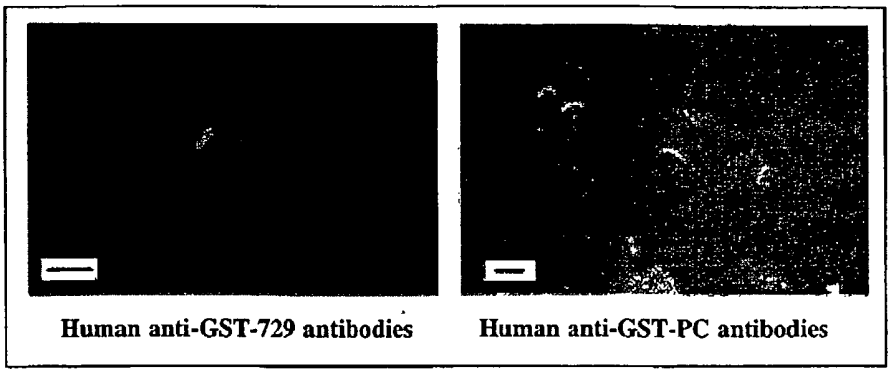
A. RT-PCR



B. WESTERN BLOT ANALYSIS



C. Immunofluorescence Antibody Test



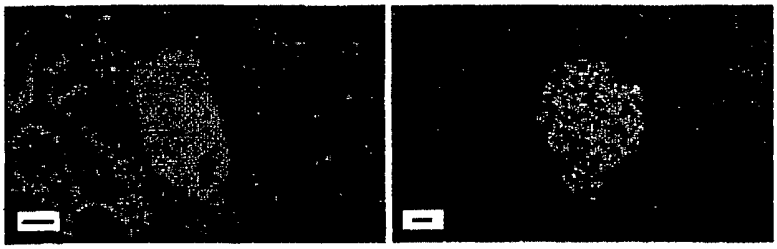
Human anti-GST-729 antibodies

Human anti-GST-PC antibodies

A. Due to the difficulties in obtaining an adequate quantity of sporozoite mRNA, Northern blot analysis could not be performed at this stage and transcription of *lsa-3* gene was studied by RT-PCR. Oligonucleotides i1 (+) and i2 (-), located 3' of exon 1 and 5' of exon 2 respectively, allowed amplification of the expected 125 bp fragment in NF54 mRNA (lane 2) whereas control DNA (lane 3) and contaminating DNA (lane 2) gave a 293 bp band. Lanes 1: 100 bp ladder (Amersham). Effective splicing of the intron were further confirmed by subcloning of the 125 bp fragment and complete sequencing.

B. Western blot analysis of protein extracts from uninfected mosquito salivary glands, NF54 sporozoites and blood stages (r: rings, s: schizonts) using mouse antisera directed against C) control GST recombinant protein, 1) GST-PC recombinant protein, 2) oligonucleotides GP5-GP6-GP8-GP11, 3) GST-729 recombinant protein (see Methods). In sporozoites, LSA-3 is visualized as a 175 kDa protein (*), in agreement with LSA-3 theoretical molecular weight calculated (for NF54 sequence) with the PEPTIDEMASS program (Wilkins *et al.*, 1997 and http://www.expasy.ch/tools/peptide-mass.html). **C.** By IFAT, LSA-3 appears to be located in some areas of the membrane and to distribute over the cytoplasm of sporozoites. Bars correspond to 10 µm.

Immunofluorescence Antibody Test



mouse anti-NR2 peptide antibodies
Five day-old liver schizonts (*Cebus*)

chimpanzee anti-NR2 peptide antibodies
Six day-old liver schizonts (chimpanzee)

By IFAT, LSA-3 appears located in the parasitophorous vacuole of trophozoites and in the pseudocytomere, i.e. the fluffy material surrounding merozoites from mature liver schizonts. Bars correspond to 20 µm.

RT-PCR, Northern blot, Western Blot: not accessible

Northern blot . negative (DNA probes: DG729 and PC insert; data not shown)

Western blot (see sporozoite Western blot for comparison with sporozoite and control extracts run in parallel)
. negative on extracts from all forms when using mouse antisera directed against peptides GP5-GP6-GP8-GP11 (see Methods) and GST-PC recombinant protein
. cross-reactions observed on ring and schizont extracts when using human and/or mouse antibodies directed against R2 repeats (anti-GST-729, -GST-NN and -RE antibodies)

IFAT . negative on all blood stage forms with antibodies against NR2 peptide and GST-PC recombinant (not shown)
. cross-reactions observed on rings and schizonts with human and mouse antibodies directed against R1-R2 repeats (anti-GST-729, -GST-NN and -RE antibodies; not shown)

HOMOLOGIES

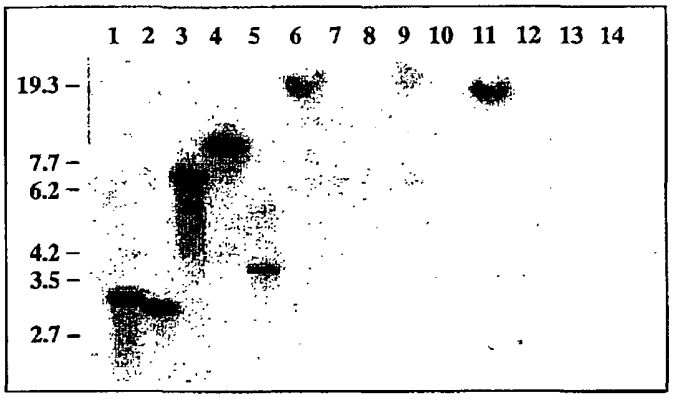
INTRA-SPECIES / INTER-SPECIES

Data banks screening (GenBank, EMBL and SwissProt) with LSA-3 non-repeated sequences did not reveal any significant homology (>30%) with other known genes or proteins. As expected from their high valine and glutamine content, R2 repeated sequences did show significant homologies with PfRESA repeats, expressed at the surface of infected-erythrocytes and a member of the *P. falciparum* glutamic acid-rich antigenic network which also comprises antigens Pf11.1 and Ag332 (Moelans & Schoenmakers, 1992).

***lsa-3* gene is a single-copy gene in *P. falciparum* genome.**

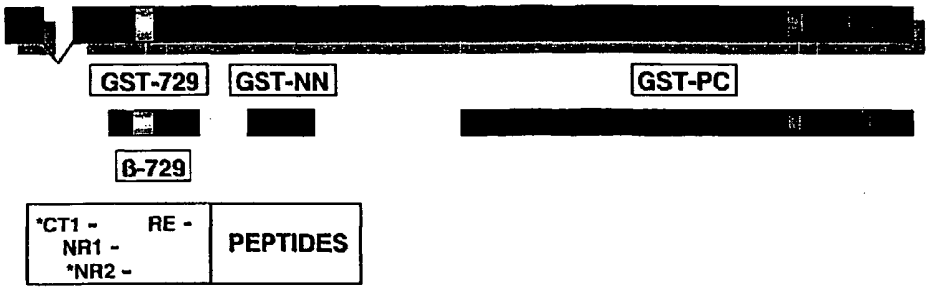
A single band, corresponding to a single-copy gene, is observed below in each of lanes 1-4 where a DG729 DNA probe was hybridized at low-stringency with (see section "Restriction map") *Sca I/Eco* RI (lanes 1-2) and *Eco* RI/*Hin*d III (lanes 3-4)-digested *P. falciparum* DNA from NF54 (lanes 1, 3) and T9/96 (lanes 2, 4) parasites.

The same experiment performed with *Eco* RI/*Hin*d III-digested DNA from *Plasmodium gallinaceum* (lane 7), *vivax* (lane 8), *knowlesi* (lane 9), *cynomolgi* (lane 10), *chabaubi* (lane 12), *yoelii* (lane 13) and *berghei* (lane 14) did not reveal any homologous sequences in these species, except in the simian parasite *P. reichenowi* (lane 5) which is closely related to *P. falciparum*. Lanes 6 and 11: molecular weight markers.



SYNTHETIC PEPTIDES & RECOMBINANT PROTEINS

used for chimpanzee immunisations



SYNTHETIC PEPTIDES

. **CT1**	aa 140-159	LLSNIEEPKENIIDNLLNNI
. **NR1**	aa 177-201	DELFNELLNSVDVNGEVKENILEES
. **NR2**	aa 198-223	LEESQVNDDIFNSLVKSVQQEQQHNV
. **RE**	derived from block R2 of clone DG729	VESVAPSVEESVAPSVEESVAENVEESV

*: for the immunisations, CT1 and NR2 were also employed as palmitoyl-conjugated lipopeptides prepared as described in Ben Mohamed *et al.* (1997).

RECOMBINANT PROTEINS

. **ß-729**: ß-galactosidasefused protein encoded by the DG729 insert (aa 1'-150'), cloned in frame within the *Eco* RI site of the phage λgt11

. **GST-729**: GST (glutathione-S-transferase)-fused protein encoded by the DG729 insert (aa 1'-150'), cloned in frame within the *Eco* RI site of pGEX.A plasmid (Invitrogen)

. **GST-NN**: GST-fused protein (aa 369-447) encoded by the klenow filled-in *Nla*IV-*Nla*IV restriction fragment (nucl.1269-1509, K1 strain), cloned in frame within the *Sma*I site of pGEX-2T plasmid (Invitrogen)

. **GST-PC**: GST-fused protein (aa 869-1786) encoded by the klenow filled-in *Pvu*II-*Cla*I restriction fragment (nucl.2768-5574, K1 strain), cloned in frame within the klenow filled-in *Eco* RI site of pGEX-3X plasmid (Invitrogen)

METHODS

1. Parasites

[0083] Blood stages of *P. falciparum* T9/96 clone (Thaithong *et al.*, 1984), NF54 (Ponnudurai et *al.*, 19881) and K1 (Thaithong and Beale, 1981) strains were cultured as described by Trager and Jensen (1976). *P. falciparum* sporozoites were obtained from NF54 strain as described in Ponnodurai *et al.* (1989) and from mosquitoes fed with gametocytes produced *in vitro* from Thai patient isolates (Galey et al., 1990). *P. falciparum* liver schizonts were identified in liver biopsies of a Sapajou monkey (*Cebus apella*, in day 5 post-sporozoite challenge) infected with the African isolate 730XI (Druilhe *et al.*, 1984), and of a chimpanzee (*Pan troglodytes*, in day 6 post-sporozoite challenge) infected with NF54 strain (Meis *et al.*, 1990).

2. Nucleic acid isolation and hybridisation

[0084] Parasite genomic DNA was purified from saponin-lysed infected erythrocytes (Robson *et al.*, 1991). Total RNA from sporozoites and parasite blood stages were extracted according to Chomczynski *et al.* (1987). DNA probes were randomly [^{32}P]-radiolabelled according to the manufacturer's recommendations (Amersham, UK). Southern and Northern blottings, probe hybridisations and washes were performed on 5-10 µg of material by standard methods (Sambrook *et al.*, 1989).
Low stringency cross-species hybridisations were performed overnight at 54°C in: 5x Denhardt's solution, 6x SSC buffer, 0.1 % SDS, 0.1 mg/ml sonicated salmon sperm DNA. Membranes were washed 30 min. at 54°C in 0.2X or 0.1X SSC buffer before autoradiography.

3. Cloning and sequencing protocols

[0085] A size-selected (0.5-1.5 Kb) genomic expression library was prepared in the phage λgt11 from *P. falciparum* T9/96 DNA and differentially screened with various stage-restricted sera as previously described (Guérin-Marchand *et al.*, 1987). λgt11-DG729 and -DG679 DNA were prepared from a liquid phage lysate. The gel-purified *Eco*RI inserts were cloned into plasmid pUC18 and sequenced. The DG729 insert was randomly radiolabelled and used as a probe to screen an *Eco*RI-digested genomic DNA library prepared from the K1 strain in the phage λgt10 (generously provided by G. Langsley, Pasteur Institute). Five positive clones were isolated and analysed. One of them, clone k1.2, was found to contain the largest *Eco*RI insert and was therefore chosen for subcloning and complete sequence analysis. This 6.7 Kb *Eco*RI fragment and subclones derived from it (spanning the entire insert) were cloned into pUC18. A series of Exonuclease III-digested subclones from the 1.8 Kb repeated regions R1-R2 of clone k1.2 was obtained using the Erase-a-Base Kit (Promega, U.S.A.). All clones and subclones described above were sequenced on both strands with insert flanking or internal oligonucleotidic primers using the dideoxy method (Sanger *et al.*, 1977) and the Sequenase enzyme system (United States Biochemicals Corp.).

4. PCR and RT-PCR amplifications

[0086] RT-PCR experiments were performed on 300-500 ng of total RNA (for blood stage parasites) or on the RNA pellet obtained from 10^6-10^7 NF54 sporozoites. cDNA were synthesized from 30 pmoles of primers S2(-) by the MMLV-reverse transcriptase in a final volume of 20 µl according to the manufacturer 's recommendations (Gibco-BRL). PCR reactions were carried out on 10 µl of cDNA synthesis reaction or on 1 µg of genomic DNA, according to the manufacturer 's recommendations (Amersham, UK).
For *Isa-3* amplification in human blood samples and *P. falciparum* detection in challenged chimpanzees, PCR was performed as described in Bottius *et al.* (1996) where primers described within for clone DG157 correspond to primers S1 and S2 reported here.

5. Peptides synthesis and production of recombinant proteins

[0087] Peptides and lipopeptides used for chimpanzee immunisations were synthesized as described in Ben Mohamed *et al.* (1997). All peptides and lipopeptides were purified over 90% by reversed-phase chromatography, the impurities essentially consisting in shorter sequences. Long synthetic peptides GP5 (aa 1241-1346), GP6 (aa 1143-1255), GP8 (aa 1026-1095) and GP11 (aa 840-907) were synthesized as described in Roggero *et al.* (1995); they are all located in region NR-B (strain K1), i.e. the non-repeated region of PC insert.
Recombinant protein β-729 was prepared from a liquide lysate as described in Guérin-Marchand *et al.* (1987). Control GST protein and GST-fused recombinant proteins were prepared according to the manufacturer 's recommendations

(Invitrogen) except for GST-PC which was prepared from 20 liter cultures due to poor production yields. This large scale culture was incubated until OD_{600}= 8.0; bacteria were then pelleted, lysed using a French Press and filtered before standard purification.

6. Antibodies and antisera

[0088] Human antibodies were immunopurified on recombinant proteins and peptides as previously described in Marchand & Druilhe (1990) and Brahimi *et al.* (1993), respectively. Mouse and chimpanzee anti-NR2 peptide antibodies were induced respectively in mice and in chimpanzee Gerda by lipopeptide NR2 injections as described in Ben Mohamed *et al.* (1997). Mouse antisera against GST-PC recombinant protein and long peptides GP5-6-8-11 (used for Western blotting) were obtained following 3 subcutaneous injections of the immunogen (100 µg) emulsified in SBAS2 adjuvant (Stoute *et al.*, 1997).

7. Western blot analysis

[0089] Proteins from intraerythrocytic parasites and sporozoites were solubilized in sodium dodecyl sulphate (SDS)-containing sample buffer, subjected to 5% SDS-polyacrylamide gel electrophoresis under reducing conditions, electroblotted onto nitrocellulose membrane and detected as described previously (Bouharoun-Tayoun & Druilhe, 1992), using mouse antibodies (at dilution 1/100). Visualisation was performed by peroxidase-conjugated goat anti-human IgG and chemoluminescence (ECL Western blotting reagents, Amersham).

8. Immunofluorescence Antibody Test (IFAT)

[0090] IFAT were performed as described previously (Druilhe *et al.*, 1986) on asynchronous erythrocytic cultures of *P. falciparum* NF54 strain, on freshly dissected live sporozoites labelled in suspension, on wet sporozoites deposited on poly-L-lysine-coated slides and on glutaraldehyde-fixed sporozoites, as well as on Carnoy-fixed liver schizonts. Positive IFAT on liver schizonts were verified by phase contrast microscopy and subsequent Giemsa staining of the sections (Druilhe *et al.*, 1984).

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Annex to the application documents - subsequently filed sequences listing

[0091]

SEQUENCE LISTING

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Claims

1. A vaccine composition comprising a Th1-inducing adjuvant in combination with a protecting Liver Stage Antigen or immunological fragment thereof of a human malaria parasite with the proviso that when the immunological fragment is an immunological fragment of LSA-3, the Th1-inducing adjuvant is not Montanide.

2. A vaccine composition as claimed in claim 1 wherein the human malaria parasite is Plasmodium falciparum.

3. A vaccine composition as claimed in claim 1 or claim 2 in which the Thl-inducing adjuvant comprises QS21, De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition: a metabolisable oil, such a squalene, alpha tocopherol and tween 80.

4. A vaccine composition as claimed in claim 1 or 2 or claim 3 wherein said protecting Liver Stage Antigen is the Liver Stage Antigen 3 (LSA-3) or immunological fragment thereof.

5. A vaccine composition according to any one of claims 1 to 4 comprising in addition at least one other protecting antigen or an immunological fragment thereof, of a malaria parasite.

6. A vaccine composition as claimed in claim 4 in which the other malaria antigen is selected from the following group:

   a) a hybrid protein comprising substantially all the C-terminal portion of the CS protein, four or more tandem repeats of the immunodominant region, and the surface antigen from hepatitis B virus (HBsAg), in particular RTS,S, or immunogenic derivatives including fragments thereof;
   b) the TRAP protein of the T9/96 isolate of Plasmodium falciparum and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof;
   c) the MSP-1 of Plasmodium falciparum or Plasmodium vivax and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof; and
   d) the MSP-3 of Plasmodium falciparum or Plasmodium vivax and proteins having at least 70% homology with the C-terminal region thereof, and immunogenic derivatives including fragments thereof.

7. A vaccine composition according to claims 1 to 6 capable of involving a T cell response in a mammal to the antigen or antigenic composition

8. A vaccine composition according to claims 1 to 7 capable of stimulating interferon γ production.

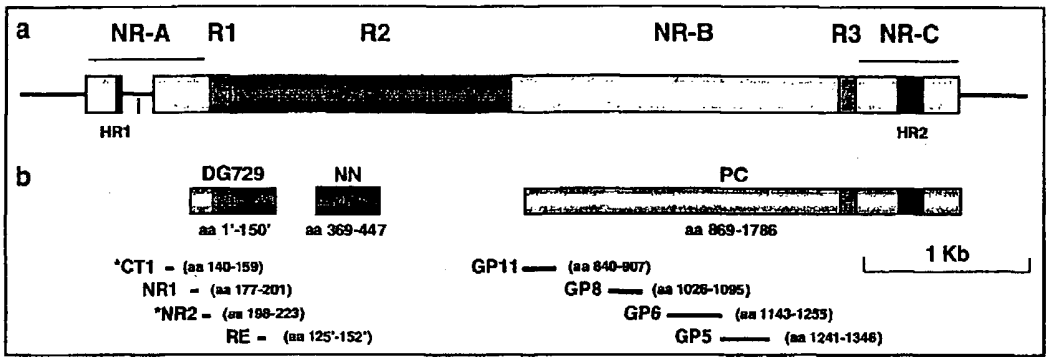
9. A vaccine composition according to claims 1 to 8, wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.

10. A vaccine composition according to claims 1 to 8, wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.

11. A process to make a vaccine composition according to any one of claims 1 to 10 comprising admixing QS21, 3D-MPL and the oil in water emulsion as defined in claim 2 with a protecting Liver Stage Antigen of a human malaria parasite.

12. A process according to claim 11 wherein the Liver Stage Antigen is LSA-3 of Plasmodium falciparum or immunological fragment thereof.

13. Use of a composition according to any one of claims 1 to 10 for the prophylaxis or treatment of malaria infections.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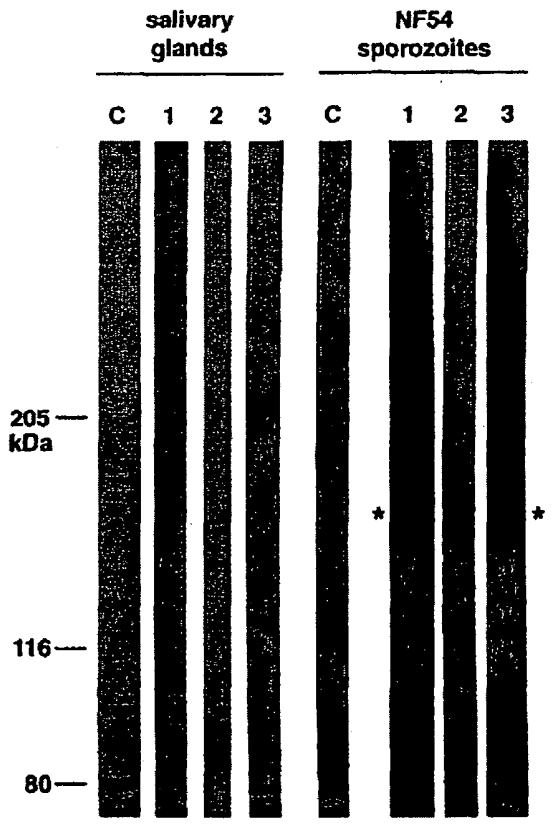

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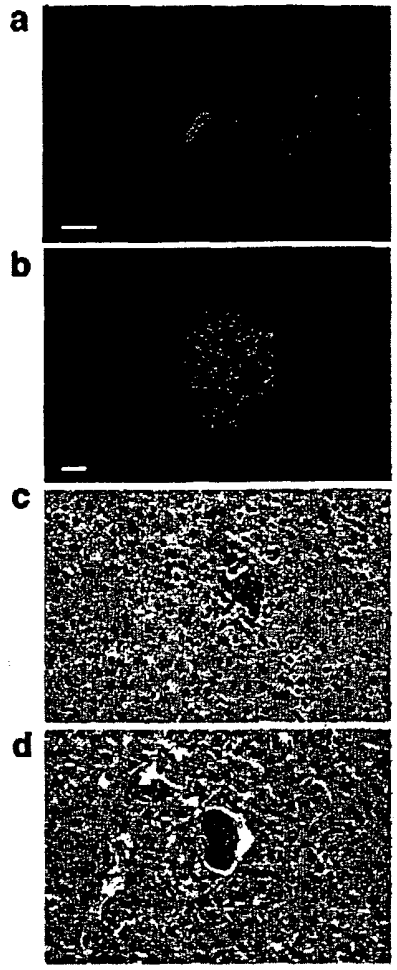

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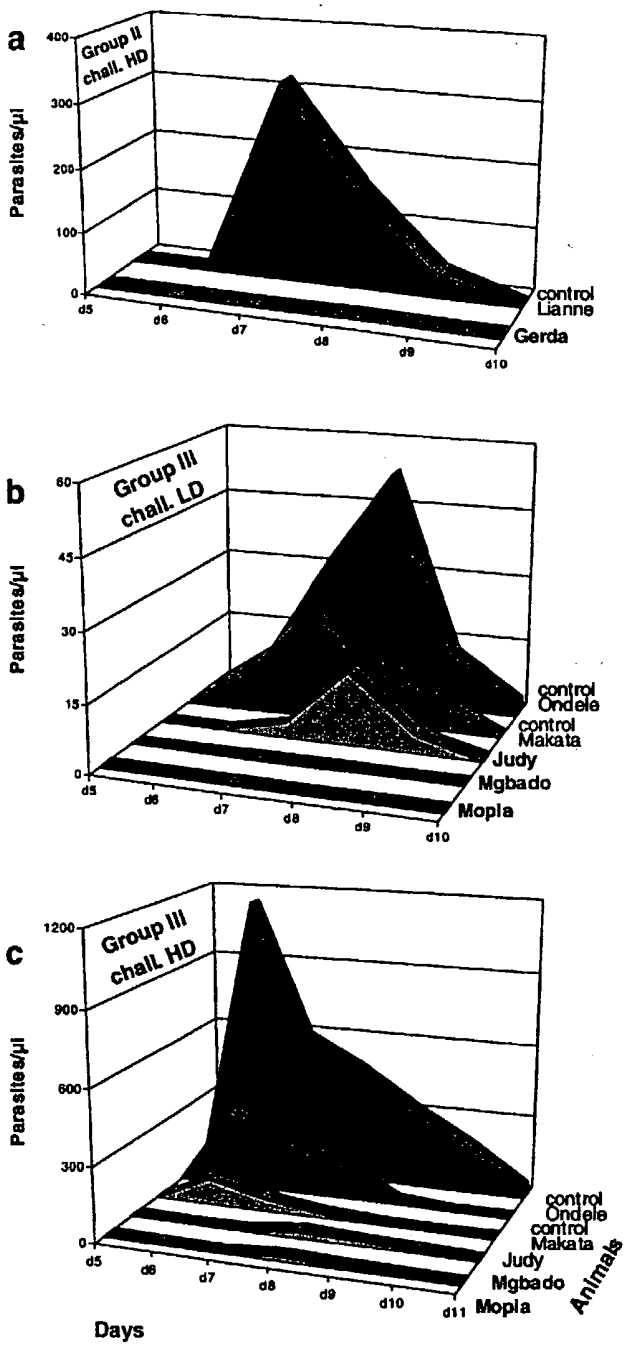
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Figure 4

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 00 20 3724

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X,D	BENMOHAMED LBACHIR ET AL: "High immunogenicity in chimpanzees of peptides and lipopeptides derived from four new Plasmodium falciparum pre-erythrocytic molecules." VACCINE, vol. 18, no. 25, 2000, pages 2843-2855, XP004203575 ISSN: 0264-410X	1,2,4,5, 7,8,13	A61K39/39 A61K39/015
Y	* the whole document *	3,6,9-12	
X	US 5 602 031 A (MARCHAND CLAUDINE ET AL) 11 February 1997 (1997-02-11) * column 2, line 24 - line 29 * * column 3, line 4 - line 22 * * column 7, line 48 - line 57 * * column 8, line 25 - line 31 *	1,2,7,13	
	-/--		

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

A61K

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

Although claim 13 is directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Place of search	Date of completion of the search	Examiner
THE HAGUE	3 August 2001	Noë, V

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C07)

European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 00 20 3724

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
Y,D	WO 95 17210 A (SMITHKLINE BEECHAM BIOLOG ;MOMIN PATRICIA MARIE (BE); GARCON NATHA) 29 June 1995 (1995-06-29) * page 1, line 3 - line 10 * * page 1, line 24 - line 34 * * page 2, line 34 - page 3, line 3 * * page 4, line 26 - line 34 * * page 5, line 4 - line 16 * * page 6, line 4 - line 16 * * page 6, line 36 - line 37 * * page 7, line 16 - line 37 * * example 5 * * claims 1-6,8-10,12,13 *	3,6,9-12	
Y	EP 0 761 231 A (SMITHKLINE BEECHAM BIOLOG) 12 March 1997 (1997-03-12) * abstract * * page 2, line 3 - line 27 * * page 2, line 52 - line 56 * * page 3, line 38 - line 49 * * page 3, line 56 * * claims 1-6,89,10,12; examples 1,2 *	3,6,9-12	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	US 5 811 106 A (HALL JENNIFER RUTH SADLER ET AL) 22 September 1998 (1998-09-22) * the whole document *	6	
A,D	US 6 017 538 A (DRUILHE PIERRE ET AL) 25 January 2000 (2000-01-25) * the whole document *	6	
A,D	US 4 837 016 A (FREEMAN ROBERT R ET AL) 6 June 1989 (1989-06-06) * the whole document *	6	

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03-08-2001

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